5-Lipoxygenase (5-LOX)-5-Lipoxygenase Activating Protein (FLAP)-Nanodisc Complex: A Model for the 5-LOX-FLAP Interaction

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A Dissertation

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Doctor of Philosophy

in

The Department of Biological Sciences

by

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I dedicate this work to my parents, Donna and Jared Schexnaydre
And to
Donovan McClelland
Whose love and support
Made this work possible.
Preface

This is a representation of my journey as a graduate student that has defined not only the type of research I desire to pursue but also the type of scientist I plan to exemplify. The journey has not been without its challenges, however with each victory over a challenge I have gained the understanding and experience needed to promote my career as a successful scientist.

When I first started graduate school I never imagined I would take on a completely new technique that was not established at my university, attend courses to learn about it, and organize and hold a workshop at the university to promote it. It started when I was developing the question I was going to address as part of graduate research. The question involved investigating the structure and function of a protein-protein-membrane complex. The interaction between this membrane-associated protein and transmembrane protein had not been observed in vitro and I endeavored to take advantage of the Nanodisc technology which allowed analysis of this complex in a membrane environment. However, the structural analysis appeared to lay outside the scope of the primary technique of the lab, x-ray crystallography. Obtaining a crystal of this irregular shaped protein-protein-membrane complex would be quite challenging. After doing some extensive research, I came across the technique of cryo-electron microscopy (cryo-EM). Single-particle cryo-EM provided the opportunity of structural analysis of a single particle which not only did not require crystallization of the particle but kept the particle in a hydrated native-like state. I was completely overtaken by the possibilities this technique could provide and started investigating the feasibility of analyzing the complex with cryo-EM. The current research demonstrated that analysis of complex structures using nanodiscs had been done before but the size of this complex was near the size limit of this technique. Although this would be a challenge, it did not make it impossible. With the support of my advisor, I participated in a three-dimensional microscopy course where I obtained a complete grasp of what pursuing cryo-EM would entail. It was an unbelievable experience and I was so captivated by this technique that I decided to promote it at Louisiana State University by organizing and holding the 3D Cryo-Workshop. This provided its own unique experiences for me. I learned how to obtain funding to support the workshop, bring in speakers that would provide excellent examples of what has been achieved with cryo-EM, organize and run both theoretical and practical sessions of the workshop as well as how to organize a committee and delegate work to others. Since then I have applied both negative stain and cryo-EM to analyzing this protein-protein-membrane complex and have formed a collaboration at Baylor of School of Medicine to obtain high quality cryo-EM imaging of this complex. As I developed the methods for reconstituting this complex in vitro and imaging it with TEM, I gained advice and perspective on what to do to push my project forward by participating in Three-Dimensional Electron Microscopy Gordon Conferences. It is at this conference that I was able to network with other people in the field and obtain encouragement and support of my project. It was with this support that my project kept driving forward.

Although significant progress has been made, the journey still continues as I optimize the cryo-EM imaging of my sample. This work could have not been possible without the continuous support of my advisor, Dr. Marcia Newcomer. I hope that you enjoy what this work has to offer.
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Abstract

Leukotrienes (LT) are lipid mediators of the inflammatory response that play important roles in diseases such as asthma and atherosclerosis. Leukotriene A4 (LTA4) is synthesized from arachidonic acid (AA) by 5-lipoxygenase (5-LOX) with the help of 5-lipoxygenase activating protein (FLAP), a trimeric nuclear transmembrane protein. Exactly how 5-LOX and FLAP interact is not well understood, however FLAP is essential for the production of leukotrienes in vivo. I used nanodiscs (NDs), engineered membrane systems, as a tool to study the 5-LOX-FLAP interaction. FLAP was incorporated into NDs with a phospholipid composition to mimic the nuclear membrane. Structural features were observed with single-particle negative stain transmission electron microscopy (TEM). This is the first in vitro reconstitution of this protein-protein-membrane interaction.

I demonstrated that a distinct 5-LOX sequence motif (tri-lysine motif) modulates activity and confers dependence on FLAP. Substitution of motif with a highly conserved sequence found in homologous enzymes results in ~20-fold higher 5-LOX product levels in stimulated HEK cells relative to the wild-type-expressing cells. However, despite the fact that the presence of FLAP increases product levels in wild-type-expressing cells, it significantly reduces the amount of product detected in cells which express the mutant enzyme. This data suggests that the tri-lysine motif limits enzyme activity and that this inhibition must be compensated by the presence of FLAP for effective LT biosynthesis.

5-LOX activity is modulated by natural products. The frankincense derived compound 3-Acetyl-11-Keto-beta Boswellic Acid (AKBA) is a well-known inhibitor of 5-LOX. We investigated the mechanism of inhibition of 5-LOX by AKBA and defined a novel allosteric binding site. In the presence of FLAP, AKBA effectively blocks the initiation of LT production in HEK cells. Data from immuno-fluorescence microscopy and in vitro studies suggest that AKBA interferes with the 5-LOX:FLAP interaction. Combined, these studies support a specific 5-LOX-FLAP-membrane interaction and provide a path to define the molecular details of this interaction.
Introduction

The Inflammatory Response as Part of the Innate Immune Response

The innate immune response is the first line of defense against foreign invaders for any organism. It is responsible for neutralizing the majority of foreign bodies that attempt to enter the human body. This defense system ranges from physical barriers such as the skin to cellular and chemical components such as specific immune cells and the chemical mediators they produce(1). The innate immune response is known for its non-specific nature of action but it can recognize pathogens with what are called pattern-recognition receptors (PRRs), which include Toll-like receptors as well as cytoplasmic receptors and they recognize patterns on foreign invaders called pathogen-associated molecular patterns (PAMPs)(2). A significant part of the innate immune response is the inflammatory response. It can be initiated as both a response to a pathogen invasion or a response to injury to healthy tissue or cells(1,3). There are five cardinal signs of inflammation: rubor (redness), tumor (swelling), calor (heat), dolor (pain) and functio laesa (loss of function). The redness and heat is due to vasodilation and an increase in blood flow to the site while swelling is due to the increased vascular permeability. The pain is a consequence of pressure build-up on nerve endings due to the swelling(4,5). All of these actions raised to an overwhelming level can cause loss of function.

Calcium has a significant role in the inflammatory response and permits the release of histamine from mast cells(1). While this process depends on the extracellular concentration of calcium, other inflammatory pathways create an influx of intracellular calcium which triggers the production of pro-inflammatory mediators(6,7). There are several cell types that participate in the inflammatory response. These include white blood cells known as leukocytes, which includes cell types such as monocytes, neutrophils or macrophages. Other cells include dendritic cells, mast cells, platelets, and lymphocytes. The main cell recruited to the site of the origin of inflammation is the neutrophil, otherwise known as polymorphonuclear cells for their multi-lobed nuclei(8). Neutrophils are the most abundant white blood cell in blood circulation(8). Macrophages are another cell type that can be commonly found at a site of inflammation though while in the resting state their numbers are usually low. Macrophages are mainly known for phagocytosis of pathogens or dead cells and tissue, but they are also involved in wound repair through the release of anti-inflammatory mediators (3).

The main objective of the inflammatory response is to amplify signals through recognition and recruitment. It does this through many different types of chemical mediators, one type being lipid mediators such as leukotrienes (LTs)(1). These mediators are important for enabling the first line of defense against pathogens and are powerful but short-lived, which is fitting for a response that has the potential to damage healthy tissue. The production of other pro-inflammatory mediators called prostaglandins and thromboxanes are inhibited by Ibuprofen or Aspirin, otherwise known as nonsteroidal anti-inflammatory drugs (NAIDs), which non-selectively inhibit the enzymes cyclooxygenase-1 and -2 (COX-1/-2)(9). These pro-inflammatory mediators as well as leukotrienes are derived from an omega-6 fatty acid called arachidonic acid (AA)(10,11). This polyunsaturated fatty acid (PUFA) is the precursor of inflammatory eicosanoids which include signaling molecules such as leukotrienes.
Leukotriene Biosynthesis

Leukotrienes are polyunsaturated lipid mediators of the inflammatory response as part of the innate immune response. Leukotrienes include LTA$_4$, an unstable epoxide that is the precursor to LTs, LTB$_4$, as well as LTC$_4$, LTD$_4$ and LTE$_4$, which are known as the cysteinyl-LTs (cysLTs). LTB$_4$ is synthesized mainly in neutrophils and it is a strong chemoattractant which recruits more neutrophils to the site of inflammation (6,12). CysLTs on the other hand increase vascular permeability and vasodilation, which can cause the bronchoconstriction and mucus secretion exhibited by asthma patients (6,12). These signaling molecules bind specific receptors that trigger the manifestation of these symptoms. The specific receptors known as BLT and CysLT receptors are G-protein-coupled receptors for which these molecules bind at nanomolar concentrations (7,13,14). Excessive release of these mediators triggers over-inflammation which is detrimental to the surrounding tissue. LTs have been exhibited in diseases such as asthma and atherosclerosis. In asthma LTs act as strong chemoattractants and cause bronchoconstriction. LTs have been shown to be released from mast cells during an asthma attack (15). Asthma is suggested to affect around one sixteenth of the people around the world. The main treatment is still corticosteroids, which only reduce some of the symptoms of asthma (6). However, corticosteroids only suppress the effects of the white blood cell, the eosinophil and not neutrophils; therefore they do not inhibit LT biosynthesis (7). Furthermore, it has been demonstrated that they cause an increase in the expression of an important protein in LT biosynthesis called 5-lipoxygenase activating protein (FLAP) (7). The initiator of LT biosynthesis is in a family of enzymes called lipoxygenases (LOXs) and requires the help of FLAP (16,17).

LOXs are enzymes that contain a non-heme iron and metabolize polyunsaturated fatty acid substrates. They are not universally expressed in yeast or bacteria, but they are found in animals as well as plants and fungi (18). There are six isoforms of lipoxygenases in humans. These isoforms include 5-lipoxygenase (5-LOX), 15-lipoxygenase-1 (15-LOX1), 15-lipoxygenase-2 (15-LOX2), 12/12R-lipoxygenase (12/12R-LOX), and epidermis-type lipoxygenase 3 (eLOX3). All of the human LOX genes are found in the same region on chromosome 17 except for 5-LOX, which is located on chromosome 10 (19). The 15-LOX1 and 15-LOX2 enzymes have been suggested to play a role in formation of atherosclerotic plaque formation by having the capacity to oxidize the lipids that make up low density lipoproteins (LDLs) (20, 21). While the 5-LOX enzyme has been shown to contribute to the bronchoconstriction in asthma, as well as arterial wall inflammation (22).

5-Lipoxygenase (5-LOX)

5-lipoxygenase is the initiator of leukotriene biosynthesis in that it catalyzes the first reaction of arachidonic acid (AA) to leukotriene (LTA$_4$) (23, 24). 5-LOX translocates to the nuclear membrane upon an increase in intracellular Ca$^{2+}$ concentration (25, 26), along with an enzyme called phospholipase A$_2$ (PLA$_2$). PLA$_2$ cleaves arachidonic acid (AA) from phospholipids in the nuclear membrane so that it is then available for 5-LOX to be converted to LTA$_4$. It converts AA to LTA$_4$ in a two-step reaction that first converts AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by first a hydrogen abstraction followed by an oxygenation and through an additional hydrogen abstraction 5-HPETE is converted to LTA$_4$ (27, 28). Although 5-LOX has the same general fold of regulatory domain and catalytic domain as other LOXs (29), it has some unique structural features that have been attributed to its unique phenotypical display. Of the LOXs it is the most structurally unstable and has a very short half-life (30, 31). 5-LOX is known to require a helper protein, FLAP a nuclear transmembrane protein which binds AA in vivo, to obtain endogenous AA for LT biosynthesis (32).
The helper protein FLAP enables 5-LOX to access substrate and promotes completion of the two-step transformation of AA to LTA₄ (16,17,33,34).

5-LOX’s expression in macrophages indicates a connection of 5-LOX’s inflammatory mediation to enhancing atherosclerosis (35). 5-LOX’s role of converting AA into LTs suggest its significance in the inflammation that promotes the accumulation of atherosclerotic plaques. One study in mice demonstrated the significance of the 5-LOX gene by comparing 5-LOX expression levels in mice resistant to atherosclerosis to a strain that did not have resistance (36). The resistant mice demonstrated a significant reduction in 5-LOX and 5-LOX products compared to non-resistant mice(36). Furthermore, 5-LOX post-translational modifications have prominent roles in the function of 5-LOX. While phosphorylation of 5-LOX at Ser-271 by MAP kinases promotes the activity of 5-LOX to produce LTs (37,38), phosphorylation of Ser-663 may have a different role. A point mutation that mimicked the phosphorylation of Ser-663 demonstrated reduced LT production, however, it exhibited lipoxin production, an anti-inflammatory compound normally produced by the combined activities of 5-LOX and 15-LOX (39). 15-LOX and 5-LOX are normally expressed in different cells. The LTA₄ produced in neutrophils is transferred to platelet cells to be picked up by 15-LOX/12-LOX to produce lipoxins (40). Phosphorylation of 5-LOX at Ser-663 may present a way to bypass this mechanism. Many factors in 5-LOX’s regulation play a role in contributing to atherosclerosis. One study found that 5-LOX gene promoter variations enhanced the development of atherosclerosis in carotid arteries (41). Overall, links between inflammation and atherosclerosis have been established, which suggests that 5-LOX and FLAP have a crucial role in atherosclerosis susceptibility.

Like other LOXs, 5-LOX contains a small regulatory domain and large catalytic domain (42) The regulatory domain binds Ca²⁺ which triggers 5-LOX to translocate to the nuclear membrane upon an influx of Ca²⁺ in the cell (43-45). The catalytic domain holds the non-heme iron coordinated by three histidine residues as well as the main-chain carboxyl of the C-terminal residue, isoleucine (42).

One unique 5-LOX feature is in αhelix2. Where other LOXs have six to seven turns in this helix, 5-LOX has only three (42). Additionally, this helix is bent ~45° from other LOXs’ αhelix2 (42). The result of this is that two residues (F177 and Y181) point down towards the active site blocking substrate access(46). These residues have been shown to be important for product specificity by allowing correctly orientated substrate entrance into the active site(46). It is suggested that unlike other LOXs that AA enters carboxyl end first in order for the pentadiene attack to take place at the right carbon of AA(29).

Another unique structural feature is a triple lysine motif located at the C-terminus of the enzyme just 20 amino acids upstream of the carboxyl end that makes up the catalytic center of 5-LOX. In this motif, KKK663-665, the lysine-665 is a leucine, in all other LOXs the substitution of the Leucine would break salt links and pi-cation interactions observed in other LOXs(42). The mutation of this triple lysine motif to ENL, in a form of 5-LOX that lacks membrane binding loops and two cysteines, increases the melting point and prolongs the half-life of this variant allowing crystallization and structural determination of the 5-LOX enzyme(42). This motif was suggested to be an auto-inactivation mechanism and therefore a key regulator of 5-LOX activity. In my work investigating this motif, a more intricate intrinsic regulation by this motif was revealed: it confers 5-LOX’s dependence on the helper protein, FLAP.
5-Lipoxygenase Activating Protein (FLAP)

In contrast to other members of the LOX family, 5-LOX requires a nuclear transmembrane helper protein (FLAP) in order to effectively transform its substrate AA (33). Upon Ca^{2+} stimulation 5-LOX co-localizes with FLAP at the nuclear membrane and several studies argue strongly for an interaction between the two proteins (47-49). The structure of FLAP with an inhibitor-bound was solved to 4.0Å(34). Trimeric FLAP is made up of three monomers and the trimer crosses the nuclear membrane at 60Å length and 30Å width with its cytosolic loops and luminal helices(34). The inhibitor binding sites in FLAP reside in the plane of the membrane between monomers of the protein(33,34). These sites give a hint as to where AA could be held by FLAP.

FLAP is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily. Another member of the MAPEG family is involved in LT biosynthesis. This enzyme is leukotriene C4 synthase (LTC4S), which is also a nuclear transmembrane protein. It catalyzes the conversion of LTA_4 to LTC_4 in conjunction with glutathione(GSH)(50). Unlike all other members of the MAPEG family, FLAP does not bind GSH nor does it have any known activity, which puts it in a subclass of its own(51,52).

The effects of overproduction of 5-LOX-generated lipid mediators are exhibited in pathologies such as asthma and atherosclerosis and it is known that 5-LOX depends on FLAP for LT biosynthesis. However, little is known about how FLAP hands off substrate to 5-LOX and the interaction between the two has been strongly suggested but not structurally observed. 5-LOX’s unique dependence on a helper protein makes the study of the 5-LOX-FLAP interaction of great interest as a possible drug target for a member of a group of enzymes that have the same overall fold and catalytic centers, and bind the same substrate.

5-LOX-FLAP Interaction

An interaction between 5-LOX and FLAP has been strongly suggested but has never been observed. With immuno-fluorescence microscopy it has been demonstrated that 5-LOX and FLAP colocalize at the nuclear membrane in HEK293 cells. Although fluorescence microscopy put 5-LOX and FLAP in close proximity to each other it was with a study by Gerstmeier et al that used proximity ligation assays to determine that 5-LOX and FLAP were within a 40nm distance of each other(49). Additionally, they demonstrated that in HEK cells FLAP rescued 5-LOX mutants' function with impaired nuclear localization and activity (49). One example depicting the importance of investigating the interaction between 5-LOX and FLAP is a study done in mice with atherosclerosis that demonstrated that the FLAP inhibitor MK-886 lead to a decrease in plaque formation(33).

In my thesis work I have demonstrated the first in vitro reconstitution of the 5-LOX-FLAP interaction. This was made possible through the use of engineered membrane systems called nanodiscs (NDs). NDs are small scale bilayers that are held together by two amphipathic membrane scaffolding proteins (MSP)(53-55). Bacterial overexpression and purification of 5-LOX and FLAP, His and Strep tagged, respectively, were applied in the reconstitution. By incorporating FLAP into nanodiscs, the ND supported a native-like environment for 5-LOX and FLAP to interact. Additionally, the nanodiscs were constructed with lipids that mimic those found in the nuclear membrane.

The purpose of my study of the interaction between 5-LOX-FLAP is to define a model for this interaction in the presence of a bilayer, which is supported by the generation of LTs. The
generation of LTs by this *in vitro* protein-protein-membrane complex sheds light on the function of the membrane in initiating reaction of LT biosynthesis. Additionally, product analysis of this complex allows us to investigate how FLAP holds the intermediate product (5-HPETE) in the active site so that 5-LOX completes the two-step reaction. The technique I have chosen to use to evaluate the 5-LOX-FLAP interaction and to observe just how FLAP interacts with 5-LOX is single-particle cryo-electron microscopy (cryo-EM). The individual crystal-structures of FLAP and 5-LOX enable the cryo-EM analysis of the complex to be even more powerful. Since the interactions between 5-LOX and FLAP and the interactions of each with the bilayer could be subtle, it makes the complex a subject for cryo-EM analysis. The cryo-EM complex data and the x-ray crystallography data of each protein provides a chance to hypothesize about the function and regulation of LT biosynthesis in diseases. Moreover, knowledge of the 5-LOX-FLAP-membrane interaction can help in the development of new ideas into analysis of this unique drug target.
I. Single-Particle Cryo-Electron Microscopy

Single-Particle cryo-electron microscopy (Cryo-EM) is a structural biology technique used to analyze the three-dimensional structure of in vitro single particles such as proteins, protein complexes, protein-rRNA molecules like the ribosome(56-58), as well as helical structures such as chromatin fibers(59) and DNA-protein complexes(60). These particles are imaged in a frozen hydrated environment which keeps them in a more native-like state. Traditional EM uses heavy negative stains such as uranyl acetate or uranyl formate, which add contrast to the sample, but at the same time dehydrate the sample and can cause an altered three-dimensional structure of the particle. In this frozen hydrated state, the particles are ideally in different orientations. The images taken from the EM (micrographs) contain two-dimensional particles that can be picked out and placed into classes based on their orientation as demonstrated by the single particle of the alcohol oxidase (AOX) enzyme (61)(Figure 1). These two-dimensional classes are then independently averaged and these averages are then used to reconstruct a three-dimensional representation of the particle. Cryo-EM, unlike the traditional method of obtaining macromolecular structures by x-ray-crystallography, is analyzed directly by imaging instead of analysis of a diffraction pattern from a crystal. However, each technique aims to obtain a three-dimensional representation of a molecular particle of interest.

In order to comprehend the applications of x-ray crystallography and cryo-EM in today’s structural biology field, we must start from the beginning. However, let’s first define x-ray
crystallography and cryo-EM. Both techniques allow a researcher to reconstruct a three-dimensional representation of a molecule such as a protein or a complex of proteins. For x-ray crystallography, a researcher must identify the ideal conditions that will crystalize his or her protein. This crystal contains identical repeating units made up of the protein and therefore locks the protein in one conformation while also amplifying the information that will be extracted from the crystal (Figure 2). Once the protein is crystalized it is hit with a beam of x-rays usually supplied by a synchrotron facility. These x-rays scatter off the crystal in a pattern that is representative of the molecule making up that crystal. Computational approaches then take the diffraction pattern created by the scattered x-rays and calculate a three-dimensional electron density map from which a molecular model is constructed and made consistent with the density map. In contrast, cryo-TEM is able to image proteins or complexes of proteins in a frozen state using electrons in a similar way light is used in a light microscope. Each image of a protein or a complex of proteins is termed a single-particle, however, unlike in x-ray crystallography these particles can be in a variety of orientations and conformations. These different conformations are separated into groups where several particle images of each group are averaged together to obtain structural details. These two-dimensional images from several different viewpoints are then computationally reconstructed into a three-dimensional structure as seen for the Methanococcus maripaludis chaperonin (Mm-cpn) (Figure 3) (62).

Although electron microscopy in today’s structural biology field of single-particle three-dimensional reconstruction is considered a “naïve technique” compared to x-ray crystallography, the origins of each developed around the same time. Röntgen first discovered X-
rays in 1895 and J.J. Thomson discovered electrons in 1897. As we begin to learn more about these two, we see that they share the property of acting as both a particle and a wave, which is the same way light functions. To understand this link and how it is applied in future explorations of science we must go back even further to the first applications of light that enabled us to see the unseen.

The Nimrud lens was the first step taken to see beyond what could be seen by the naked eye. Around 710 BC this lens, which was comprised of crystal rock, was used as a magnifying glass. The invention of the first microscopes in 16th century and their development throughout the 17th century lead to monumental discoveries such as the first observations of cells. While microscopes were developing so were scientists' understanding of crystals. It started in 1611 with the German mathematician, Johannes Kepler's analysis of the six-cornered snowflake, which lead to Xliels Stensen's Law of the Constancy of Interfacial Angles in 1670. In 1784 came Abbé Haüy's Laws of symmetry, rational intercepts, and of constancy of crystalline form. Then in 1830 German physician, John Fredrich Christen Hessel concluded that morphological forms come together to give exactly thirty-two kinds of crystal symmetry. This was followed by the 1848 discovery of fourteen unique lattices (Bravais lattices) and 230 possible space groups in 1891.

With Louis de Brogile's discovery of the particle and wave functions of an electron and Hans Busch’s discovery that a magnetic lens could direct electrons, Ernst Ruska and Max Knoll applied light optics theory to electrons by designing the first electron microscope (Figure 4). At around the same time Bragg's particle and Barkla's wave function discovery of x-rays, as well as the development of the understanding of the nature of crystals, lead to Max von Laue's invention of using crystals as diffraction grating for x-rays and to the first x-ray crystal structure, which was of salt (NaCl), the very thing today's protein x-ray crystallographers do not want to see in their crystal's diffraction pattern.

Both techniques aim to see structural details at the molecular level but each takes different paths to get there. EM was first able to capture images of organelles in a section of a cell and then with
improvements to the microscope and to sample preparation one is able to obtain high enough resolution images in order to reconstruct three-dimensional models of macromolecules (See Timeline). X-ray crystallography started with application to small structures, then more complex chemical structures and finally DNA and proteins (See Timeline). These two techniques have been around for relatively the same amount of time, however EM initially examined larger biological structures on the cellular level (smaller when compared to achievements of light microscopes) and then pushed the limit to obtain single-particle images. X-ray crystallography was first applied by analyzing crystals of salt and then to analyzing crystal structures of proteins. X-ray crystallography was driven by chemistry and cryo-EM was driven by biology, while both have roots in physics.

TIMELINE: Microscopy and X-Ray Crystallography through Time. First Intact Cell TEM Image(63); 3DTEM Tail of bacteriophage T4 (64); 3D Cryo-EM of Bacteriophage T4 (65)

Since the take-off of these techniques, both have evolved to answer the complex biological questions posed by researchers today. In the same way that people across the world can be so different but fundamentally the same, so are the techniques of cryo-EM and x-ray crystallography. People learn different approaches to living life the same way these techniques have developed to address scientific structural questions. For example, the stereotypical French
person is known to be laid back while stereotypical Germans are known for their strong work ethic. Neither person has a better approach to living life than the other, however both have their advantages and disadvantages. In turn, these different approaches each highlight distinctive aspects of human life. In the same way, cryo-EM and x-ray crystallography approach structural biological questions in different ways, therefore, allowing a researcher to choose the best technique suited to address his or her structural question. This choice is dependent on the advantages and disadvantages of each technique as well as the nature of the question being posed by the researcher. X-ray crystallography is better suited for small molecules that are easy to crystallize, while cryo-TEM is better suited for larger molecules or complexes of molecules that can be detected by a TEM. Additionally, x-ray crystallography is capable of giving a researcher high-resolution structural detail while cryo-EM’s vitrified hydrated samples allow a researcher to observe native-like information from his or her sample. The overlap between these two techniques has increased with the rapidly evolving advancements in cryo-EM. These advancements span from the microscope body itself to the imaging cameras and even to the computational three-dimensional reconstruction software. For many scientists, including myself, it is not a question of either, but taking advantage of both techniques to obtain both detailed structural information as well as overall biological information. It is like looking at the earth from outer space, but instead of just seeing the different continents you also see the exact locations where communities of people have settled to live. Both of these perspectives are needed to make conclusions about human life on earth. Therefore, it is the complementary aspect of these techniques that allows information to come together to create a biological story.

In my graduate work with cryo-EM I have analyzed the single particle of a protein complex (S-LOX-FLAP) in a membrane environment. Next, I will take you through the process of prepping a single-particle biological sample for imaging in the microscope to analyzing the micrographs for the single-particles and three-dimensional reconstruction of the particle.

Sample Preparation

Negative Stain Preparation

Once a complex is reconstituted it is first analyzed by negative stain EM to observe the homogeneity of the sample and successful interaction of the components. Negative stain analysis samples are applied to a 300 mesh Formvar and carbon coated copper grid (Figure 5) after it had been glow discharged for 30 seconds using a 12mA current. The thin carbon film is normally hydrophobic and glow discharging the grids makes them hydrophilic. When the grid is glow discharge

Figure 5. 300 mesh FCF copper grid. Source: EM Solutions
discharged it is put in a chamber with a partial vacuum where the air inside the chamber is
ionized when an electron potential is created by a high voltage that is applied between a cathode
and an anode. The negatively charged ions are deposited onto the carbon film making it
temporarily hydrophilic(66). During glow discharge the ionized gas in the chamber forms a
plasma which is seen as a purple glow (Figure. 6). When a sample is applied to a grid that is not
glow discharged it will bead up on the grid and the sample will not be optimally deposited on the
grid, but when glow discharged the sample spreads out on the grid.

Before imaging the grid a heavy metal negative stain must be applied in order to add contrast to
the sample. In electron microscopy, exposure of a sample to a high energy electron beam
impacts the sample and energy is absorbed by the sample. Biological samples, made principally
of carbon, oxygen, nitrogen, and hydrogen, are not very electron dense and therefore do not
absorb this energy well. Negative stain adds the density needed to provide contrast to the
sample. After negative staining, the grids can be directly imaged in the microscope; however, a
more intricate process is needed for cryo sample preparation. Single particle negative stain
imaging is predominately used for preliminary imaging for homogeneity of the particle as well as
uniform complex formation. However, negative stain has been used to analyze single particles for
structural details as done in a study by Burgess et al. that examined the structures of the motor
proteins, myosin and dynein and aligned and averaged negative stain particles for two-
dimensional details (67). Other work such as that done with anthrax toxin pore achieved a three-
dimensional structure of a negative stained particle(68) which was also solved with cryo-EM(69).

Cryo-Plunge Freezing

For single-particle samples that will be prepared for cryo-EM imaging you would use what are
called Holey grids. In these grids each square contains rows of empty holes surrounded by a
carbon support film. The grids are glow discharged the same as the carbon coated copper grids.
The grid is held by a special pair of forceps that attach into a cryo-plunge apparatus. The
chamber containing the grid has a humidity control so that when the sample is applied it
minimizes the evaporation rate of water from the grid, which would otherwise concentrate the
sample. The sample is applied and mechanically blotted for a preset number of seconds before
being plunged into liquid ethane where it is instantaneously frozen. This liquid ethane is
prepared by cooling ethane gas with liquid nitrogen so that it state transitions to a liquid. The
melting temperature of liquid ethane is -182 °C and it is used over liquid nitrogen (melting
temperature: - 209 °C ) because of its higher heat capacity which allows the sample to be frozen
more rapidly (70). When the sample is blotted on the grid a thin layer of the sample fills the holes.

Figure 6. Leica EM ACE 600 plasma glow from glow-discharging
Subsequently, when the grid is plunge frozen into the liquid ethane the thin layer of liquid sample becomes a thin layer of vitrified sample, meaning that the sample was frozen so fast that it does not allow the formation of ice crystals (Figure 7). This allows for optimal imaging of the imbedded particle because ice crystals damage the particle and would disrupt imaging and they can be seen very well in the microscope. Once the sample on the grid is frozen in liquid ethane it must be transferred to liquid nitrogen and placed in a small grid storage box. This step is one of the critical points where the grid can be subject to what is called ice contamination, meaning moisture from the air crystalizes on the grid and thus potentially ruins the sample. From here the grid storage box must be transferred to a liquid nitrogen storage container until it is ready to be imaged in the microscope.

**Imaging**

The Transmission Electron Microscope (TEM)

An electron microscope is composed of an electron source, electromagnetic lenses, a specimen holder, and detectors, as well as additional components such as an energy filter and phase plate.

![Figure 7. Vitrification of cryo sample in liquid ethane. Source: Gabriel Lander](image)

**Figure 8. JEOL 1400 TEM Source: EM Core Facility at the University of Missouri-Columbia.**
The electron source is made up of a filament such as tungsten or lanthanum hexaboride (LaB6) or a field emission gun (FEG). Tungsten and LaB6 are thermal filaments, meaning heat is applied to the filament which causes the emission of electrons. The FEG applies a strong electrical field near the filament which causes electrons to tunnel out of the filament. FEG supplies a steadier and more power beam than the tungsten and LaB6 filaments and is commonly found in high resolution cryo-TEMs. Electromagnetic lenses are coils of copper wire inside iron pole pieces and the current through the coils creates a magnetic field. There are several electromagnetic lenses within the microscope which include a condenser lens, an objective lens, an intermediate lens, and a projector lens. The specimen holder is partially immersed in the bore of the objective lens due to the small focal length of the lens. Detectors consist of a fluorescent screen where a specimen can be viewed directly, as well as cameras which can be either charged coupled devices (CCDs), which indirectly detects electrons, or direct detection devices (DDDs), which directly detect electrons.

In negative stain imaging the prepared grid can be directly placed into a specimen holder that inserts into the microscope. The microscope is under a vacuum so the specimen holder is first inserted into a specimen chamber, pumped to the vacuum of the microscope before hand and then fully inserted such that the specimen lies in a small space between the condenser lens and objective aperture lens. In order to start imaging, the high voltage that supplies the electron beam needs to be turned on. Once the sample is inserted, the beam is turned on and the grid can be immediately visualized on a fluorescent screen where initial focusing takes place. Once initial focusing is done on the fluorescent screen, finer focusing of the sample is done using a camera. This can be in the form of a CCD camera or DDD camera. For high resolution imaging of particles, a direct detector is optimal.

In order to understand why direct electron detectors are an innovation in single-particle cryo-EM, we must first look back at how electron micrograph image detection has evolved over time. The first form of detection of electron micrographs for TEM was halide emulsion film. The electrons from the TEM would hit the halide and convert it into silver, which could be developed with various chemicals to extract an image. EM films have a wide field of view, high contrast and give a high signal to noise ratio, which is described as the detective quantum efficiency (DQE). However, film requires a long exposure time and it is a tedious process developing the film. Nevertheless, it is interesting to note that the first TEM 3D structure was reconstructed with film micrographs (64). A major breakthrough came with the ability to use digital cameras for EM. This camera is the CCD camera, which uses a scintillator to detect electrons and convert them to a photon signal through a fiber optic network and then back into an electrical signal to be viewed by imaging software (Figure. 9). Not only was it easier to acquire images, but also CCDs do not

![Figure 9. CCD camera detection (left) versus DDD camera (right) detection](source: 3D Microscopy Course-Winter school at the University of Zurich)
require a long exposure time. However, the resolution as described by the modulation transfer function (MTF) can be lower than film when collecting high quality micrographs for 3D reconstruction. The MTF evaluates the difference in the input and the output signal in terms of spatial frequency. The MTF of a CCD camera is dependent on the pixel size and the quality of the fiber optics. Since the electrons initially detected are converted into photons and then back into electrons, information can be lost in the process. When comparing imaging of amorphous carbon film with both a CCD camera and film, it was found that CCD cameras were better at detecting low resolution data while film was better at detecting high resolution data (71). What was needed was a camera that could be used to collect high-resolution data in a short exposure time.

This camera is the direct electron detection camera, which as its name states directly detects electrons therefore eliminating the conversion process. Direct electron detectors are based on Monolithic Active Pixel Sensor (MAPS) technology (72) and designed by the Complementary Metal-Oxide Semiconductor (CMOS) method. Direct electron detectors are composed of pixel transistors and photodiodes on top a p-epitaxial layer (73). When electrons hit this sensor the electrons generate more electrons by ionization, with each electron producing several ionization electrons. The electrons are collected by each pixel and put into a digital format. These direct detectors read out data at a much higher frame per second (fps) rate than CCD cameras. The UltraScan 4000 indirect detector reads at 0.2 fps with a 15μm pixel size while the DUOS integrating direct detector has a 20μm pixel size functioning at 30fps, however it has a smaller full frame readout (74). The K2 Summit in counting mode has a 5μm pixel size and runs at 400fps with a 3X larger full frame than the DUOS and 7X larger full frame when functioning in super-resolution counting mode (74). The differences in the K2 Summit’s different modes of detection are featured in Figure 10.

Figure 10. Direct electron detection. Source: 3D Microscopy Course-Winter school at the University of Zurich

in Figure 10. The advancement of direct electron detectors has been fueled by the drive to push the resolution limit of structural reconstruction of macromolecules with cryo-EM. The year 2015 was a flourishing year for cryo-EM with over 100 molecular structures solved with the technique (75). The advancement of direct electron detectors has significantly contributed to this success and has allowed cryo-EM to become a competitor to x-ray crystallography.

Unlike with negative stain imaging, cryo sample grids must be kept at liquid nitrogen temperatures during transfer to the microscope, as well as while imaging. Before imaging cryo samples, the microscope must be cooled down with liquid nitrogen, then the grids stored in liquid nitrogen must be seated into a cryo specimen holder (Figure. 11), which has a mini dewar attached, and vacuum must be applied the day before. This is another critical step that is prone to ice contamination. The mini dewar keeps the sample cold while in the microscope. Once placed in the microscope the grids are imaged under a low electron dose. This dose ranges from 10-20 e-/Å² while in traditional negative imaging it can be up to 50-100 electrons/pixel (67,76). One area
on the grid is sacrificed as the focusing area, meaning that the sample there is too damaged due to the time the beam has impacted the area as well as the slightly higher electron dose used in focusing. Using what is called a minimal dose system (MDS), the microscope switches to another hole for acquisition once the sample is in the optimal focus (77). When the cryo sample is completely in focus you will not see the particles because they do not have contrast. In order to gain contrast, the sample must be slightly defocused typically around 0.5-3.0 µm at the cost of resolution in the process (78). From here micrographs can be taken for analysis and 100s to 1000s of micrographs can be taken and single particles analyzed.

![Figure 11. Gatan Cryo-holder](image)

There are a few components to an electron microscope that can potentially help the quality of the micrographs. High resolution cryo-EM are usually 300kV microscopes, meaning that they have a higher operating voltage over the 120 and 200kV microscopes. One accessory is an energy filter which is a component that uses electron energy loss spectrometry (EELS). It works by evaluating the energy distribution of electrons and removing the electrons that were inelastically scattered from the sample (79). This filtering process helps reduce background noise and also improves image amplitude contrast (79,80). Another component is a phase plate to give phase contrast when imaging. This is normally obtained by defocusing, which is good for getting high spatial frequency information but low partial frequency information. However, phase plates allow for a large range of spatial frequency information (81). A phase plate is placed after the objective lens in the diffraction plane and acts by changing the normally sine curve seen in the contrast transfer function (CTF) to a cosine curve (82). The CTF is a mathematical representation of much of the phase contrast of the sample is transferred to the observed image in terms of spatial frequency. The CTF is best described by Joachim Frank as “The description of the relationship between observed image contrast and projected object potential, and the way this relationship is influenced by electron optical parameters, is the subject of the contrast transfer theory” (83).

There are two main different types of phase plates, the Zernike phase plate (ZPP) and the Volta phase plate (VPP). The ZPP is made up of amorphous carbon with a hole in the center and was the standard phase plate for a time but it had a short lifespan, however VPP is continuous carbon and can be used for years. However, because the beam passes through a carbon film some signal is lost due to the scattering of electrons and it can also introduce some astigmatism due to the uneven surface of the carbon film (81).

**Analysis**

Once micrographs of a particle are collected they are ready to be analyzed computationally for three-dimensional reconstruction. There are several single-particle reconstruction software packages available and the major ones EMAN2, RELION, and SPIDER. All of these programs perform the same basic steps but the way they go about it can be slightly different. EMAN2 is good at 2D class averaging but RELION is preferred for constructing a 3D initial model. For a given particle one may choose to do some steps in one program and perform the rest on another.
Data can be carried over during some steps from one program to another program and continued to be processed as long as the data files are in the correct format. These three software programs are open source and are accompanied with online tutorials so they are commonly used and updated by the developers as well as easy to access.

The first version of EMAN2 was released in 1999 and updated versions are released at least two times a year. EMAN2 has a C++ core library and uses Python scripting language with command lines and GUI displays for image visualization and manipulation(84). Over the years three-dimensional reconstruction programs have become more user friendly, though some understanding of the Linux environment is still required.

Single-particle cryo-EM has come a long way over the past several decades and its foundation recently celebrated with the 2017 Noble Prize in Chemistry for single-particle cryo-EM by Richard Henderson, Joachim Frank, and Jacques Dubochet. The evolution of the attainable resolution of some protein structures has come a long way in cryo-EM. For example, GroEL chaperonin complexes, a 10-12 subunit complex with 57kDa monomers, were first negatively stained in 1979 and viewed just as single-particle images(85) and later a low resolution three-dimensional structure at 11.5Å was produced in 2001(86). The latest structure in 2017 has been solved to a resolution of 3.5Å(87)(Figure. 12). This is just one of many structures that serve as an example for how much the field of single-particle cryo-EM has grown over the past several decades, especially from the early 2000s until now. It continues to grow with the new advancements in imaging and detection technology.

Figure 12: GroEL Structure. Left. negative stain EM images Source: (85). Top right. 11.5Å structure Source: (86). Bottom right. 3.5Å structure Source: (87).
II. 5-Lipoxygenase (5-LOX)-5-Lipoxygenase Activating Protein (FLAP) Nanodisc Complex

The 5-LOX enzyme and transmembrane protein FLAP have been strongly suggested to interact and there is evidence for 5-LOX’s dependence on FLAP(33,49). Purified detergent solubilized FLAP has not been demonstrated to interact with purified 5-LOX, suggesting that the membrane bilayer plays a crucial role in the interaction between 5-LOX and FLAP. The interaction of 5-LOX and FLAP has not been reconstituted in vitro until now with this work presented here. Single-particle cryo-EM and other biochemical techniques have been used to analyze the assembly of the 5-LOX-FLAP-ND interaction.

Nanodiscs

Nanodiscs are engineered membrane systems consisting of phospholipids encased by a membrane scaffold protein (MSP) (54,55,88,89). Nanodiscs provide membrane proteins with a native-like environment and cryo-EM structures of the ribosome-SecYE complex (58), the anthrax toxin translocon-lethal factor complex (69), and intact human integrin αIIbβ3 (90) have all been reported with nanodisc constructions. Cryo-EM structures enable larger overall structural and functional features to be observed in a macromolecular complex. For example, an F-type ATP synthase (91) complex provided an overall understanding of how individual components work together, while the fine details are examined by the individual component x-ray crystal structures docked into the complex structure. This same approach will be taken with the 5-LOX-FLAP-nanodisc complex.

Nanodiscs were constructed to mimic the lipid composition of the nuclear membrane. The nuclear envelope lipid composition is not well defined but it has been characterized based on the lipid make-up of the ER membrane, which is a continuation of the nuclear membrane (92). Based on the overall lipid composition of leukocytes, nanodiscs were designed with approximately 75% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 25% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and 2% 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC). Nanodiscs enable reconstruction of a single-particle system by cryo-EM imaging without the need to control the direction of insertion of FLAP into the bilayer, as either side will be

![Figure 13. Micrographs of Empty Nanodiscs. Left. Negative stained nanodiscs. Right. Cryo-EM of nanodiscs.](image)
accessible to 5-LOX. The specified phospholipids and MSP are solubilized in a solution containing cholate detergent. A longer MSP belt (MSP1E3D1) was expressed and purified, which gives ~129Å diameter nanodiscs (55). Once constructed, the nanodiscs with Histidine-tagged MSP are purified by cobalt affinity chromatography. The homogeneity of nanodiscs is then analyzed by negative stain EM and further analysis with cryo-EM (Figure. 13). The specific nanodiscs I have constructed provide an environment that approximates FLAP's native environment. Please refer to the Materials and Methods section for a detailed outline of the nanodisc construction process.

**FLAP Incorporated Nanodiscs**

FLAP-incorporated nanodiscs were constructed using MSP1E3D1 and a mixture of the three classes of phospholipids. The phospholipid mixture and MSP are solubilized in a cholate detergent buffer. FLAP is overexpressed and purified from *E. coli* in n-dodecyl-β-D-maltoside (DDM) detergent buffer to keep it soluble. To confirm successful FLAP incorporation, FLAP nanodiscs were immuno-gold labeled and imaged by negative stain EM (Figure. 14). As expected empty nanodiscs and FLAP nanodiscs do not differ in size. Since FLAP is expressed with a Histidine-tag, FLAP occupied nanodiscs were purified on cobalt-affinity column. The histidine-tag on MSP was cleaved off by incubation with the tobacco etch virus (TEV) protease, which specifically recognizes a sequence of seven amino acids that allows removal of affinity tags. Negative staining revealed views of FLAP-nanodiscs; FLAP can be seen as a single dark impression located in the center of the nanodisc (Figure. 15). Nanodiscs provide a native-like environment for studying FLAP's interaction with 5-LOX and are applicable to single-particle TEM.

Nanodiscs have been shown to maintain the active form of the protein embedded in it, especially those that require particular lipids for their function (53). The incorporation of FLAP into nanodiscs is accomplished during nanodisc self-assembly when detergent is removed. FLAP is added to the cholate solution of lipids and MSP, therefore there is a mixture of detergents that are
slowly removed by bio-beads for nanodisc formation. This mixture of detergents helps stabilize the protein more so than cholate alone. If a membrane protein is exposed to cholate for too long it will compromise the integrity of the protein (53,93). A single FLAP particle is embedded in a single nanodisc, as visualized by immuno-gold labeling of FLAP in FLAP-nanodiscs constructions

![Image](image_url)

**Figure 15.** Negative stain EM imaging of FLAP incorporated nanodiscs. Above. FLAP-ND single particle images. Right. FLAP-ND micrograph.

in the EM. Though the FLAP trimer is suspected to be incorporated in the nanodiscs, it is possible that it is just a monomer of FLAP. Due to the close proximity of antibody epitope binding sites it may not be possible to bind more than one FLAP monomer at a time. Therefore, the FLAP trimer is suspected to be what is observed in the EM.

**5-LOX-FLAP-ND Interaction**

The structure of 5-LOX-FLAP-nanodiscs will be determined by single-particle cryo-EM. I have started a collaborative project with the EM National Center for Macromolecular Imaging (NCMI)

![Image](image_url)

**Figure 16.** Model of the 5-LOX-FLAP interaction.
facility at Baylor School of Medicine. All preliminary images were taken with a JEOL 1400 microscope at the LSU Shared Instrumentation Facility. Once optimal micrographs are obtained, they will be analyzed with three-dimensional reconstruction software, EMAN2/RELION (84,94) that is currently running on of the supercomputers at LSU’s high-performance computing center, remotely accessed with a lab computer. Once the complex structure is solved, the individually known x-ray crystal structures of 5-LOX and FLAP will be docked in to the complex. This will allow in- and out- of complex molecular differences to be analyzed, therefore providing the details of the role of FLAP in the regulation of 5-LOX. A working model for how 5-LOX might bind to FLAP embedded in a nanodisc bilayer is depicted in Figure 16. In this model the 5-LOX membrane insertion loops are positioned to insert into the bilayer. The active sites of the two components align for efficient substrate transfer.

Although cryo-EM is a new technique to Louisiana State University (LSU), we have an electron microscope with cryo capabilities that can be used to acquire sufficient preliminary images. The collaboration with the EM center at Baylor provides us access to a state-of-the-art detector. To gain a better understanding of this technique I attended a three-dimensional microscopy course at the University of Zurich, where I learned the theoretical and practical aspects of cryo-EM. In order to spread awareness of what can be achieved with this technique, I organized a cryo-EM

Figure 17. Negative stain EM of 5-LOX-FLAP-ND. Left. Micrograph of 5-LOX-FLAP-ND with example of the complex indicated in the black circle. Above. An enlarged image of the complex in the black circle. The 5-LOX enzymes traced in orange and one measured to 8.9nm. Based on the crystal structure of 5-LOX 8.9nm is the expected length of 5-LOX. FLAP-Side view of FLAP-ND is traced in yellow.
workshop at LSU. The workshop included outside speakers who are experts in the field. Along with the speakers, I lectured on the basic principles of this technique and provided hands-on sessions to familiarize interested scientists on what goes into cryo-EM. Single-particle cryo-EM is my main focus in examining the complex structure of 5-LOX and FLAP in a membrane environment.

Wild-type 5-LOX was overexpressed in *E. coli* and purified with a modified protocol that was modeled after the work by *Kumar et al* (95). 5-LOX is a challenging enzyme to purify and stabilize; determination of the 5-LOX structure, required several mutations in order to purify and crystallize a stable but functional enzyme (42). Wild-type 5-LOX’s short half-life along with its association with membrane make it a difficult enzyme to isolate in active form. However, with the addition of catalase and FeSO₄ to protect the non-heme iron (95), and working quickly at cold temperatures, I was able to purify 2.4 mgs of active enzyme from a 3 L culture, a yield which is suitable for single-particle cryo-EM as well as functional assays.

Purified 5-LOX was incubated with the FLAP-nanodiscs, and since 5-LOX readily associates with membrane, it has an affinity for FLAP-nanodiscs. Preliminary images of 5-LOX-FLAP-NDs were analyzed with negative stain EM (Figure. 17). Although there is still crowding of the complex that needs to be worked out, the 5-LOX-FLAP-ND can be seen coming together as indicated by the black circle in the micrograph. The complex indicated in the black circle can be seen as a single FLAP-ND with what appears to be two 5-LOX enzymes. When compared to the crystal structure of 5-LOX, the shape and length measurement of the particle at 8.9 nm is a good indicator that it is indeed 5-LOX interacting with the FLAP-ND. This interaction is also supported by the fact that clustering observed with 5-LOX-FLAP-ND is not seen with 5-LOX-Empty-ND (Figure. 18). Both

![Figure 18. Negative stain EM 5-LOX-Empty-ND. Right. Micrograph of 5-LOX-Empty-ND. Above. Enlarged image of 5-LOX and Empty-NDs indicated in the black circle.](image)

micrographs of 5-LOX-FLAP-ND and 5-LOX-Empty-ND were analyzed in the absence of Ca²⁺. However, the addition of Ca²⁺ has been shown to cause stacking of NDs (96) and it is likely that
clustering would be seen by both 5-LOX-FLAP-ND and 5-LOX-Empty-ND in the presence of Ca\(^{2+}\). This suggests that the clustering of the 5-LOX-FLAP-ND seen in Figure 17 is due to the attraction between 5-LOX and FLAP.

The interaction of 5-LOX and FLAP was also analyzed by clear native polyacrylamide gel electrophoresis (PAGE) and western blot analysis (Figure. 19). The fading of the FLAP-ND band with increasing ratio of 5-LOX to FLAP-ND in the anti-FLAP western blot suggests an affinity of the two proteins for each other and is consistent with the presence of FLAP-ND in the native gel. The amount of FLAP-ND was kept constant while increasing amounts of 5-LOX were added to the gel (Figure. 19). Based on previous evidence of LOX's dependence on Ca\(^{2+}\) to bind nanodiscs (95,97), I examined 5-LOX's dependency on Ca\(^{2+}\) for nanodisc association. Although immuno-fluorescence imaging of 5-LOX in HEK293 demonstrated 5-LOX’s dependence on Ca\(^{2+}\) for nuclear membrane association, 5-LOX-FLAP-nanodiscs analyzed in the presence and absence of Ca\(^{2+}\) by size exclusion chromatography (SEC) demonstrated similar binding affinity though it may be possible that trace amounts of Ca\(^{2+}\) were still present.

![Figure 19. Native PAGE and Western blot analysis of 5-LOX-FLAP-ND. Left. Native PAGE stained with Sypro Ruby. The first lane is protein marker (not well stained by Sypro ruby) followed by purified 5-LOX, purified FLAP, Empty-ND, FLAP-ND, 5-LOX-Empty-ND then 5-LOX-FLAP-ND at increasing 5-LOX:FLAP-ND ratios of 0.25:1, 0.5:1, 1:1, and 2:1. Right. Western blot of native gel on the left with development with anti-FLAP antibody. The FLAP-ND band is seen to diminish with the increasing ratio of 5-LOX: FLAP.](image)

AA was introduced in the nanodisc self-assembly process and preliminary data of product generation by 5-LOX-FLAP-NDs and 5-LOX-Empty-NDs was analyzed by high pressure liquid chromatography (HPLC). Only the intermediate product was observed by HPLC analysis (Figure. 20). However, the reduced intermediate product, 5-HETE was observed near the limit of detection for HPLC analysis therefore product analysis of the complex is being further analyzed by LC/MS/MS by our collaborators, the Werz lab at Jena Universität to detect possible LT products. A functional 5-LOX-FLAP-nanodisc interaction should increase the amount of LTA\(_4\) (at the expense of the intermediate 5-HPETE). Ca\(^{2+}\)-dependent access to AA in liposomes has been demonstrated by a LOX enzyme (98). Therefore, an increased attainability of AA should be
observed with addition of Ca$^{2+}$ in 5-LOX-Empty-ND. Moreover, once 5-LOX binds to the FLAP-nanodiscs, it should obtain AA from FLAP, therefore the products observed are due to the 5-LOX-FLAP-nanodisc interactions.

Cryo-EM suits the question being addressed about the 5-LOX-FLAP-nanodisc complex. How 5-LOX and FLAP interact with each other, as well as how they both interact with the nuclear membrane may require dynamic movements that do not only have one orientation. With cryo-EM, the complex will be examined in multiple conformations. Docking of the already known individual crystal structures of 5-LOX and FLAP into this complex structure will provide both the biochemical and biological understanding needed to conceptualize the function and regulation of this complex in LT biosynthesis. I anticipate observing the general interaction sites of 5-LOX and FLAP and gain information into how FLAP contributes to leukotriene biosynthesis. With this information we could gain insight into the regulation of leukotriene biosynthesis, which will allow us to take a further look into how this pathway functions in its known associated diseases. Determining the structural basis for function and regulation of the 5-LOX-FLAP-ND complex, as well as the details of substrate acquisition by the complex, is essential for understanding its role in LT biosynthesis. Structural and functional studies of the 5-LOX-FLAP-ND complex will shed light on how the biosynthesis of leukotrienes is regulated and therefore lead to insight on how 5-LOX and FLAP contribute to these diseases. Cryo-EM complex data complemented with the x-ray crystallography data of 5-LOX and FLAP will provide an enhanced perspective of the overall mechanism of LT biosynthesis. Additionally, structural information of the 5-LOX-FLAP-ND interaction could lead to inhibition studies for this drug target.

The complementarity of the individual crystal structures of 5-LOX and FLAP with the overall complex structure and their interactions with the membrane enable us to understand the overall mechanism and regulation of LT biosynthesis. Furthermore, the individual crystal structures will provide detailed biochemical information to understand the larger conformational changes and vice versa. The resolution and size limit of single-particle cryo-EM is being pushed everyday with structures like the 95kDa TRPV1 ion channel at 3.4Å (75,99) and the 2.2Å structure of β-galactosidase (75,100). The increasing potential of single-particle cryo-EM makes determination of the structure of the 5-LOX-FLAP-ND complex possible.

Figure 20. Preliminary HPLC product analysis of 5-LOX-FLAP-ND and 5-LOX-Empty-ND
III. A Specific 5-lipoxygenase Motif Impedes Enzyme Activity and Confers Dependence on a Partner Protein

Leukotrienes (LT) are lipid mediators of the inflammatory response that play key roles in diseases such as asthma and atherosclerosis. The precursor leukotriene $A_4$ (LTA$_4$) is synthesized from arachidonic acid (AA) by 5-lipoxygenase (5-LOX), a membrane-associated enzyme, with the help of 5-lipoxygenase-activating protein (FLAP), a nuclear transmembrane protein. In lipoxygenases the main chain carboxylate of the C-terminus is a ligand for the non-heme iron and thus part of the catalytic center. I investigated the role of a lysine-rich sequence (KKK$^{653-665}$) 20 amino acids upstream of the C-terminus, unique to 5-LOX, that might disrupt placement of the main-chain carboxylate in the metal coordination sphere (Figure 21). A 5-LOX mutant in which KKK$^{653-665}$ is replaced by ENL was transfected into HEK293 cells in the absence and presence of FLAP. Substitution of KKK with ENL leads to the formation of ~20-fold higher 5-LOX product levels in stimulated HEK cells relative to the wild-type expressing cells. Co-expression with FLAP elevates

Figure 21. Structural perspective of the triple lysine motif. A. Two 5-LOX cartoon diagrams related by a 180° rotation. The “Tail” and “Body” antibody epitopes are highlighted in green and blue, respectively. The Triple Lysine mutant residues (ENL) are shown in stick rendering in magenta. B. Sequence comparison of human 5-LOX and other LOXs highlighting the conserved Arg$^{651}$ (blue) and the unique 5-LOX Lys$^{655}$ where there is a highly conserved Leu (magenta). C. The Triple Lysine Motif is located at the turn between the C-terminal helix and carboxy-terminus, which is a ligand for the catalytic non-heme iron. The “Tail” epitope (green), and the conserved Arg and Leu (blue and magenta) are depicted.
product formation of wild-type 5-LOX but dramatically decreases product formation of the mutant down to the levels of wild-type enzyme. 5-LOX product formation and enzyme levels suggest that the KKK motif limits enzyme activity and that this attenuated activity must be compensated by the presence of FLAP as a partner protein for effective LT biosynthesis.

The biosynthesis of leukotrienes (LT), lipid mediators of the inflammatory response, is initiated by 5-lipoxygenase (5-LOX) (23,24). 5-LOX translocates to the nuclear membrane upon intracellular Ca\(^{2+}\) mobilization (25,26), along with phospholipase A\(_2\) (PLA\(_2\)). PLA\(_2\) cleaves arachidonic acid (AA) from phospholipids that form the nuclear bilayer so that it is then available for 5-LOX to be converted to LTA\(_4\). Additionally, 5-LOX requires a helper protein, 5-lipoxygenase-activating protein (FLAP), a nuclear membrane-embedded AA-binding protein that facilitates substrate access and promotes completion of the two-step transformation of AA to LTA\(_4\) (16,17,33,34). This transformation proceeds \textit{via} (i) hydrogen abstraction at C7 and the oxygenation of AA at C5 and (ii) hydrogen abstraction from the 5-hydroperoxyeicosatetraenoic acid (5-HPETE) intermediate at C10 to generate LTA\(_4\) (27,28) (Figure. S1). 5-LOX and FLAP co-localize at the nuclear membrane and are suggested to interact (47,101-103).

5-LOX is a short-lived enzyme (30,31,104,105), as is fitting for an enzyme that initiates the synthesis of inflammatory compounds that activate G-protein coupled receptors at nanomolar concentrations (13,14,106). The effects of overproduction of 5-LOX-generated lipid mediators are exhibited in pathologies such as asthma and atherosclerosis. Auto-inactivation has been proposed to play an important regulatory role in temporal control of 5-LOX activity (107,108). Enzyme lability, whether a consequence of turnover or non-turnover-based inactivation, can serve as an auto-shutoff valve, an innate "intrinsic regulator." Auto-inactivation that is a consequence of protein (in)stability has been described as a mechanism for temporal control of protein function in other systems. For example, the short half-life of the activated, mature protease Thrombin-Activatable Fibrinolysis Inhibitor (TAFIa) ensures a short burst of activity. TAFI circulates as the zymogen, but once processed by thrombin, its half-life is on the order of minutes (109). This instability can be defined as a functional or structural instability of the protein. It has been observed that residues of an enzyme that are vital for its function do not contribute to the structural stability of the enzyme, rather to its structural instability. A study by Shoichet \textit{et al.} demonstrated that mutating specific residues in the active site of the T4 lysozyme depleted the enzyme's activity but increased its structural stability (110). Here we describe such a system where a specific sequence mutation incites structural stability, however only functional instability in relation to the balance of leukotriene biosynthesis. It is important to note that the overproduction of 5-LOX products can lead to the detrimental effects of over-inflammation, thus I will define functional stability in terms of functional output of the 5-LOX enzyme.

**Why a Triple Lysine Motif?**

We asked whether 5-LOX might harbor a unique structural feature in the canonical LOX fold (there are six different LOX isoforms in humans) that might provide a structural basis for its short life span and we identified a unique sequence motif (KKK\(^{653-655}\)) that confers enzyme structural instability \textit{in vitro}. Indeed, replacement of KKK\(^{653-655}\) with its counterpart from a stable 5-LOX homologue (\textit{i.e.} ENL, in an expression construct trimmed of its membrane-insertion loops) allowed us to obtain the crystal structure of so-called Stable-5-LOX (42). The structural basis for KKK-invoked instability is easy to rationalize: the presence of K655 (where LOX counterparts have a Leu) positions a charged amino acid for repulsion with the invariant Arg-651 (Figure. 21). The structure of a bacterial LOX that harbors an Arg at the Leu position confirmed this prediction: the invariant Arg that participates in a conserved salt link has been displaced in this enzyme (111).
The carboxy termini of LOXs insert into the catalytic domains so that the terminal, backbone carboxyl serves as a ligand for the catalytic iron. The $\text{KKK}_{653-655}$ sequence in 5-LOX is located in a turn 20 amino acids upstream of the carboxylate-ligand and its presence may make it difficult to insert the “tail” of the enzyme into the active site. Substitution of $\text{KKK}_{653-655}$ with ENL leads to a variant (Tri-Lysine Mutant, TKM-5-LOX) that is resistant to cleavage by chymotrypsin in vitro in conditions in which the wild-type enzyme is rapidly digested. TKM-5-LOX retains the ability to translocate to the membrane and to co-localize with FLAP in HEK cells. Of interest, we observed that HEK cells expressing TKM-5-LOX display a ~20-fold increase of 5-LOX product formation relative to wild-type enzyme. In parallel experiments with $\text{Ca}^{2+}$-ionophore-stimulated cells we see an accelerated loss of TKM-5-LOX, so the mutant enzyme is still subject to turnover-based inactivation. However, when co-expressed with FLAP, 5-LOX products detected in HEK cells for both the mutant and wild-type enzyme are equivalent, an indication that the KKK motif is not critical for modulation of 5-LOX activity by FLAP. Our data suggest that the KKK motif is a governor of 5-LOX enzyme activity; the helper protein FLAP is required to sufficiently relieve its auto-suppression for effective LT synthesis.

Results

Purified WT-5-LOX and TKM-5-LOX were subjected to limited proteolysis by chymotrypsin. The enzymes were incubated with the protease in the absence and presence of $\text{Ca}^{2+}$. TKM-5-LOX exhibited a higher resistance to cleavage by chymotrypsin than WT-5-LOX when the proteins were assessed with two distinct antibodies: one specific for the C-terminal twelve amino acids (“Tail”) and the other for amino acids 130-149 (“Body”) (Figure 22, antibody epitopes highlighted in Figure 21). Essentially, little to none of the full-length enzyme was detected by either antibody when WT-5-LOX was incubated with chymotrypsin. In contrast, TKM-5-LOX remained largely intact in these same conditions. Interestingly, $\text{Ca}^{2+}$, which binds to the amino terminal membrane-binding domain, seems to minimize the presence of a low molecular weight band derived from the C-terminus for both enzymes and to retain a trace amount of the full-length enzyme of WT-5-LOX.

Figure 22. In vitro chymotrypsin cleavage analysis. WT-5-LOX and TKM-5-LOX cleavage patterns with “Body” (left) or “Tail” (right) antibody development, +/- $\text{Ca}^{2+}$ and/or chymotrypsin. WT-5-LOX displays susceptibility to chymotrypsin cleavage, while TKM-5-LOX is strikingly more resistant. The lane containing the molecular weight markers is labelled “mwm.”

exhibited a higher resistance to cleavage by chymotrypsin than WT-5-LOX when the proteins were assessed with two distinct antibodies: one specific for the C-terminal twelve amino acids (“Tail”) and the other for amino acids 130-149 (“Body”) (Figure 22, antibody epitopes highlighted in Figure 21). Essentially, little to none of the full-length enzyme was detected by either antibody when WT-5-LOX was incubated with chymotrypsin. In contrast, TKM-5-LOX remained largely intact in these same conditions. Interestingly, $\text{Ca}^{2+}$, which binds to the amino terminal membrane-binding domain, seems to minimize the presence of a low molecular weight band derived from the C-terminus for both enzymes and to retain a trace amount of the full-length enzyme of WT-5-LOX.
The major band detected for the TKM-5-LOX after exposure to protease is likely to be full-length enzyme given that it is recognized by an antibody raised to the C-terminal peptide and migrates at the same molecular weight as the purified enzyme.

The TKM-enzyme is also more resistant to chymotrypsin in cell lysates. The lysates of HEK cell expressing WT- and TKM-5-LOX (but not FLAP) were incubated with chymotrypsin, with and without A23187 Ca\(^{2+}\)-ionophore. The products of the incubations were analyzed by Western blot using the Tail antibody. It is important to note that the cleavage bands observed in samples incubated without chymotrypsin demonstrate the enzyme's susceptibility to activated cellular proteases upon lysis of the cell as lysates did not contain protease inhibitors. Fragments detected for WT enzyme are more pronounced than in the lysates of TKM-expressing cells (Figure 23).

Immunofluorescence microscopy studies indicate that like the wild-type protein, the TKM-5-LOX variant translocates to the nuclear membrane upon stimulation with Ca\(^{2+}\) ionophore, both in the absence and presence of FLAP (Figure 24).
Detection by both Body and Tail antibody demonstrated that TKM-5-LOX colocalizes with FLAP, as seen for the wild-type enzyme (Figure. S2). Additionally, the mutant displays the same cytoplasmic localization as the wild-type when unstimulated (Figure. 24).

TKM-5-LOX was expressed in HEK293 cells at levels comparable to the WT-5-LOX, as judged by mRNA and protein levels, detected by qPCR (Figure. S3) and Western blotting, respectively (vida infra). 5-LOX-expressing HEK cells devoid of FLAP were incubated with or without Ca2+-ionophore A23187 in the presence of 3 μM AA. At this concentration of AA the bulk of the substrate is expected to partition into the bilayer and is only accessible to 5-LOX once the enzyme translocates to the membrane. Both the final LTA4 hydrolysis products and the intermediate 5-HPETE (which is detected as the reduced alcohol 5-hydroxyeicosatetraenoic acid, 5-HETE) were quantitated by high-pressure liquid chromatography (HPLC) analyses. As expected, 5-LOX product formation was only detected once cells were stimulated with Ca2+-ionophore. In the absence of FLAP, TKM-5-LOX-expressing cells yielded strikingly (~20-fold) higher overall product compared to WT-5-LOX cells (Fig. 25). However, the proportion of LTA4 hydrolysis products relative to the intermediate 5-HETE is roughly the same (~30%). This ~20-fold higher product formation is not due to elevated protein expression levels (vida infra) and indicates that TKM-5-LOX has significantly improved or prolonged enzyme activity over WT-5-LOX.

In agreement with previous findings (32), co-expression of FLAP in HEK cells together with 5-LOX caused a ~4-fold increase in product formation by the WT enzyme (Figure. 25). In contrast, co-

Figure 24. Cellular localization of 5-LOX variants in HEK 293 cells. A. WT-5-LOX and TKM-5-LOX expressing HEK cells in the absence (unstimulated) and presence (stimulated) of Ca2+-ionophore A23187, developed with “Body” or “Tail” primary antibodies. The nucleus is indicated by DAPI (blue) and 5-LOX by AlexaFluor-488 (green). In the absence of Ca2+-ionophore the enzymes are dispersed in the nucleus and cytoplasm. Once stimulated clear “rings” surround the nuclear DNA staining. B. When expressed with FLAP, both WT-5-LOX and TKM-5-LOX co-localize with FLAP in the presence of Ca2+-ionophore A23187, as detected with either “Body” or “Tail” primary antibodies. FLAP is imaged with AlexaFluor-647 (red). Combined TRITC and Cy5 channels demonstrated colocalization of 5-LOX and FLAP at the nuclear membrane as confirmed by ImageJ colocalization analysis.
expression of FLAP reduced product formation of TKM-5-LOX down to the levels of WT-5-LOX. There is, however, a difference in the relative amounts of LTA₄ hydrolysis products vs. 5-HETE detected, with the TKM-5-LOX yielding a higher ratio (~60%) of final product than WT-5-LOX (~30%). While the presence of FLAP attenuates the increased activity of TKM-5-LOX, it promotes completion of the two-step reaction catalyzed by TKM-5-LOX.

We next monitored the 5-LOX protein levels in the HEK cells in order to investigate whether the substitution of KKK with ENL conferred increased enzyme longevity, as might be inferred from enzyme decay profiles. Using a method adapted from that described by Dai et al. (112), 5-LOX protein levels (relative to the house-keeping protein GAPDH) in the presence of the protein synthesis inhibitor cycloheximide were monitored in HEK cells for four hours by Western blot. In addition, these experiments were repeated in the presence of Ca²⁺-ionophore A23187 under conditions where 5-LOX becomes activated and translocates to the nuclear membrane (Figure 26) to access free AA.

In the absence of FLAP and Ca²⁺-ionophore, the levels of WT- and TKM-5-LOX remained roughly stable up to 4 h, regardless of the presence of FLAP. In these conditions, the WT-5-LOX appears to be expressed at slightly elevated levels (Fig 26). However, upon Ca²⁺-ionophore stimulation of cells devoid of FLAP, the levels of the TKM-5-LOX rapidly declined, while that for the WT-5-LOX remained stable. Thus, the rapid disappearance of TKM-5-LOX is consistent with its enhanced enzyme activity/high turnover which contributes to the enzyme’s functional instability. In contrast, when co-expressed with FLAP, where 5-LOX product levels of WT and mutant enzyme are comparable, the levels of TKM-5-LOX remain stable.
Intrinsic Regulators

The KKK$^{653-655}$ sequence is located 20-amino acids upstream of the C-terminal Ile-673, which ligates the active site iron via its carboxyl moiety. This unique sequence motif appears to conflict with stabilizing salt-link and π-cation interactions observed in other (more stable) LOX structures. K655 of the KKK motif in other LOXs is typically a conserved Leu. A highly conserved Arg-651 lies one turn away on the C-terminal helix and participates in a conserved salt-link with Asp-473 and a cation-π interaction with Phe-469 (42). Thus, a Lys at 655 in WT-5-LOX may disrupt these stabilizing interactions and impact the positioning of the C-terminal carboxyl in the Fe$^{2+}$ coordination sphere. The mutation of the KKK to ENL was performed to stabilize 5-LOX for crystallographic studies, and it was observed that the mutation increased the melting temperature of the protein (42). This led us to suggest that the motif might act as an “intrinsic
regulator" by accelerating enzyme inactivation in the absence of substrate, as 5-LOX has an atypically short half-life compared to other LOXs (107). TKM-5-LOX is less susceptible to protease as the purified enzyme, or in crude cell lysates. However, we observed that the levels of both WT-5-LOX and TKM-5-LOX remain stable in HEK cells when substrate is not available. On the other hand, the KKK sequence significantly tempers an otherwise robust enzyme activity.

I have demonstrated that the KKK\textsuperscript{653-655} sequence is a unique 5-LOX structural feature that acts as an intrinsic regulator of 5-LOX to control the flow of LT biosynthesis. In the absence of FLAP, I observed a striking difference in the activity of the WT- and TKM-5-LOX. The KKK motif severely constrains a robust LOX activity as observed by the \( \sim 20 \)-fold increase in product formation when the KKK sequence is substituted by ENL (Fig. 25). However, this increase in activity is not apparent when TKM-5-LOX is co-expressed with FLAP. In these conditions, 5-LOX product levels of WT- and TKM-5-LOX are equivalent. While the presence of FLAP increases the LT biosynthetic capacity of the WT-5-LOX-expressing cells, the TKM-5-LOX-expressing cells display a significantly reduced LT biosynthetic efficiency when FLAP is present.

FLAP is able to compensate for the lower activity of the WT-5-LOX to some extent. Thus, we suggest that the KKK motif confers the requirement for FLAP for the rapid initiation of LT biosynthesis. This regulatory mechanism is reminiscent of the cyclin/cyclin-dependent kinase scenario, where an enzyme (the kinase) requires interaction with a binding partner to promote catalytic competence (113). Overproduction of LTs as pro-inflammatory mediators is detrimental to the organism, thus the tempered activity of 5-LOX that relies on alleviation by a protein partner limits this possibility. WT-5-LOX alone is particularly inefficient at producing lipid mediators, but stimulated by FLAP it still does not reach the levels produced by the TKM-5-LOX expressed without FLAP. Thus, we interpret TKM-5-LOX's high level of product output as a functional instability in respect to LT biosynthetic pathway efficiency. The fact that FLAP prevents the marked increase in 5-LOX product formation by TKM-5-LOX suggests that the KKK motif is not required for interaction with FLAP.

By monitoring enzyme levels in the presence of an inhibitor of protein biosynthesis, we observed turnover-dependent inactivation of 5-LOX. In the absence of Ca\textsuperscript{2+}-ionophore, both WT- and TKM-5-LOX exhibited stable protein levels. However, in the presence of Ca\textsuperscript{2+}-ionophore there is a rapid decline in the level of TKM-5-LOX in the cell (Fig. 26). The cycloheximide analysis was performed without addition of exogenous AA, however, we can detect the products of TKM-5-LOX activity (expressed in the presence of FLAP) in Ca\textsuperscript{2+}-ionophore-stimulated HEK cells without the addition of exogenous AA (Fig. S4). The presence of endogenous phospholipid-esterified AA that might provide a source of substrate is supported by data from other groups. Dawaliby et al established that HEK293 cell membranes contain C20 containing phospholipids (114). More specifically, Zhu et al (115) report the presence of 20:4n-6 fatty acids in the cell membrane phospholipids of HEK293 cells. Additionally, HEK cells transfected to express 12-LOX generate product without the addition of exogenous AA when cultured in DMEM (32). Therefore, the elevated product levels detected in HEK cells expressing TKM-5-LOX in the presence of exogenous AA and the rapid decay of TKM-5-LOX once stimulated with Ca\textsuperscript{2+}-ionophore to promote release of endogenous PL-esterified AA are consistent with turnover-based inactivation.

Other examples of auto-inactivation conferred by a specific sequence motif include one described by Fenalti et al. The flexibility of a catalytic loop in a particular isoform of glutamic acid decarboxylase (GAD65) enables the release of cofactor and therefore subsequent inactivation of the enzyme (116). Specific integral protein sequence motifs may act as a "kill switch" to allow modulation of enzyme activity. Although the KKK bears resemblance to other inactivation motifs,
in this case the effects of the "kill switch" are partially negated by a partner protein that compensates for the switch so that LT biosynthesis functions at effective levels.

The KKK motif might be an integral deactivation sequence that acts as a regulator of LT biosynthesis. Internal enzyme fail-safe mechanisms are one of many regulatory mechanisms that keep the intricate web of biosynthetic pathways in check. An intriguing auto-inactivation mechanism of another LT biosynthetic enzyme has been described for LTA₄ hydrolase. In this enzyme, the presence of Tyr-378 makes it susceptible to covalent modification by its reactive substrate. Substitution of Tyr-378 with Gln or Ala leads to a variant less susceptible to covalent modification, and consequently inactivation, by the substrate (117-119). Thus, both 5-LOX and LTA₄ hydrolase display auto-suppression of catalytic activity, but through entirely different mechanisms.

Other amino acids in 5-LOX have been demonstrated to play a significant role in tempering a robust rate of product formation. Rakonjac et al. examined the significance of a salt-link between residues R101 and D166 of the regulatory and catalytic domains, respectively. Disruption of this interaction leads to an increase in enzyme activity (120).

Our studies do not reveal exactly how the presence of the KKK motif modulates 5-LOX activity. Computational methods, as summarized by O'Rourke et al, can be combined with X-ray data to reveal possible internal protein communication networks (121). However, the lack of a high-resolution native 5-LOX structure makes this approach inaccessible at this point. In any case, it is apparent that the KKK motif significantly contributes to tight regulation of 5-LOX activity as the initiator of LT biosynthesis. This motif, which initially we studied as a possible destabilization mechanism that limits protein half-life, appears to be part of a more intricate, finely-tuned control mechanism to regulate LT biosynthesis in conjunction with FLAP.
IV. Allosteric Inhibition of 5-Lipoxygenase (5-LOX) and Impairment of Substrate Access from 5-Lipoxygenase Activating Protein (FLAP) by 11-Acetyl-keto-beta Boswellic Acid (AKBA)

Leukotrienes (LT) are lipid mediators of the inflammatory response that play key roles in diseases such as asthma and atherosclerosis. The precursor leukotriene A₄ (LTA₄) is synthesized from arachidonic acid (AA) by 5-lipoxygenase (5-LOX), a membrane-associated enzyme, with the help of 5-lipoxygenase-activating protein (FLAP), a nuclear transmembrane protein. Upon Ca²⁺ stimulation 5-LOX translocates to the nuclear membrane where the substrate-binding helper protein FLAP resides. The natural product 3-Acetyl-11-Keto-beta Boswellic Acid (AKBA), specifically found in frankincense, is a well-known non-competitive inhibitor of 5-LOX. We investigated aspects of the mechanism of inhibition of 5-LOX by AKBA and solved the first crystal structure of Stable-5-LOX in complex with an inhibitor. We define a novel allosteric binding site wedged between the membrane-binding and catalytic domains of 5-LOX, ~30 Å from the active site iron. Both in vitro and in 5-LOX expressing HEK cells, the absence of FLAP shifts the regio-specificity of 5-LOX to catalyze 12-HPETE formation at the expense of 5-HPETE. However, in the presence of FLAP, AKBA effectively blocks the initiation of leukotriene production in HEK cells. Additionally, immuno-fluorescence microscopy demonstrated a decrease in colocalization of 5-LOX and FLAP at the HEK cell nuclear membrane in the presence of AKBA, while native gel analysis of the in vitro complex of 5-LOX-FLAP-incorporated nanodisc displayed a decreased association of the two components in the presence of AKBA. This work suggests a novel approach to isoform-specific 5-LOX inhibitor development through exploitation of an allosteric site in 5-LOX which interferes with its ability to obtain its substrate from FLAP.

What Is AKBA?

The anti-inflammatory properties of frankincense have been well documented(122,123), and the active ingredient 3-Acetyl-11-Keto-beta Boswellic Acid (AKBA) was shown to be an effective non-

![Figure 27. 3.0Å resolution structure of Stable-5-LOX with AKBA bound. Top left. Cartoon rendering of structure. Bottom left. Space filling model. Top right. Chemical structure of AKBA. Bottom right. Coordination of AKBA in the allosteric binding site of Stable-5-LOX. (crystal structure solved by Nathaniel Gilbert)](image-url)
effects of AKBA on 5-LOX activity in the absence or presence of its helper protein FLAP differ, and show that AKBA may interfere with the 5-LOX-FLAP interaction at the nuclear membrane. Data obtained from 5-LOX expressing HEK cells and from polarized macrophages suggest that AKBA may provoke a shift in the regio-specificity of 5-LOX. Moreover, we describe the first co-crystal structure of an inhibitor with 5-LOX (Figure. 27). This allosteric binding site, wedged between the membrane-binding and catalytic domains, lies some 30 Å from the catalytic iron.

AKBA is an allosteric inhibitor of 5-LOX that binds in a deep groove at the interface of the membrane binding and catalytic domains. The polar groups of AKBA are positioned to H-bond with the side chains of H130, R101, and T137, and main chain atoms of E108 and V110. This model is entirely consistent with the structure activity relationship defined by Salier et al. with leukocytes (127). In their cell-free assays they demonstrated the essentiality of the C-ring keto group and A-ring carboxylate of AKBA; both groups can participate in H-bond to 5-LOX. Subsequent work from the same group demonstrated that AKBA with the A-ring acetoxy group replaced with a 4-azido-5-iodo-salicyloyl-beta-alanyl moiety can be cross-linked to 5-LOX (124). This bulky, reactive group extends AKBA lengthwise to penetrate deeper into a cave-like gap between the two domains, as the domain interface is not tightly packed (Figure. 27). Although a similar groove at the membrane-binding -catalytic domain interface is present in the homologous enzyme 15-LOX-2, each of the H-bond donors differs (Q108 for R101, Q136 for H130, A144 for T137) and the groove is constricted at the C-ring by a charge pair that spans it (R68, W140). Accordingly, we did not observe inhibition by AKBA at equivalent concentrations in HEK cells expressing 15-LOX-2 (Figure. 29).

**Results**

LTA₄ and 5-HETE production were monitored in HEK cells expressing 5-LOX or 5-LOX and FLAP(32). These artificial constructs allow us to evaluate the role of FLAP in the inhibition by

![Figure 28. Product analysis of 5-LOX in HEK293 cells. Incubations of AKBA at 0, 25, 50, 75 and 100µM. A. 5-LOX expressing HEK cells. B 5-LOX-FLAP expressing HEK cells. C. 12-HETE/5-HETE ratio of products from 5-LOX expressing cells.](image-url)
AKBA. We found that at 25 µM AKBA, 5-LOX activity was reduced by 50% in intact cells expressing both FLAP and 5-LOX, in agreement with what has been observed in crude cell-free assays (human recombinant 5-LO, leukocytes lysates IC\textsubscript{50} = 15 - 50 µM) (128)) and in intact primary neutrophils (IC\textsubscript{50} = 3.2 µM (129)). In contrast, in HEK cells expressing only 5-LOX the addition of AKBA led to increasing levels of 12-HydroxyEicosaTetraEnoic acid (12-HETE), at the expense of 5-HETE, in the HEK cells (Figure. 28,29). (The hydroperoxy products are reduced prior to HPLC analysis.) This observation suggests that AKBA only functions as a typical and potent enzyme inhibitor when 5-LOX interacts with FLAP at the nuclear membrane to obtain substrate. In addition, we asked whether AKBA might also modulate the activity of 15-LOX-2, its closest intra-species orthologue. The amount of 15-HETE detected in the extracts of 15-LOX-2 expressing cells was unchanged by the addition of AKBA at concentrations up to 100 µM (Figure. 29).

We monitored the co-localization of 5-LOX with FLAP upon Ca\textsuperscript{2+}-stimulation, which promotes 5-LOX translocation to the nuclear membrane in 5-LOX expressing HEK cells regardless of whether FLAP is co-expressed (32). At inhibitory concentrations of AKBA, immuno-fluorescence assays indicate that 5-LOX traffic to the nuclear membrane, but the overlay of FLAP and 5-LOX is qualitatively different from the overlay in the absence of AKBA. Colocalization analysis suggests a weaker correlation between the two antibodies used for detection (rabbit anti-5-LOX vs. goat anti-FLAP). This difference can be appreciated in Figure 30. The fact that AKBA-inhibition of 5-LOX is observed only in the presence of FLAP might suggest that the inhibitor functions by interfering with the enzyme’s ability to obtain its substrate from the helper protein. When FLAP is not present, the presence of 12-HETE in the HEK cell extracts suggests that AKBA does not interfere with access to substrate, but modulates the enzyme regio-specificity. i.e. 12-HETE formation is promoted at the expense of 5-HETE formation.

Additionally, chymotrypsin cleavage patterns of WT-5-LOX were monitored in vitro in the presence of AKBA. Cleavage bands were detected by two different rabbit anti-5-LOX primary antibodies. One to the surface epitope of 5-LOX called “body” antibody and one to the C-terminal end of 5-LOX called “tail” anti-body. The secondary antibody, donkey anti-rabbit Alexafluor 647 was detected with fluorescence imaging. Development with both tail and body antibodies displayed a decrease in particular 5-LOX cleavage bands with increasing concentrations of AKBA (Figure. 31) This could be attributed to a slight increase in structural
stability of 5-LOX with AKBA bound. AKBA bound between the membrane binding domain and catalytic domain may limit the flexibility that 5-LOX may need for a proper interaction with FLAP.

An in vitro 5-LOX-FLAP complex was formed by incorporation of FLAP into a membrane mimic. It has been inferred that the membrane plays an essential role in the interaction between 5-LOX and FLAP. FLAP was incorporated into the engineered membrane system known as nanodiscs. The 5-

Figure 30. Immuno-fluorescence microscopy of 5-LOX-FLAP expressing HEK293 cells. Cells were stimulated with Ca\(^{2+}\) ionophore A23187. The nucleus is indicated by DAPI. 5-LOX is demonstrated in green by a donkey anti-rabbit Alexafluor 488 and FLAP by donkey anti-goat Alexafluor 647. Consequently, imaged with TRITC and Cy5 filters. Cells were incubated with 0, 25, 50, 75 or 100µM AKBA.

Figure 31. In vitro Chymotrypsin cleavage of WT-5-LOX. Cleavage analysis of 5-LOX in the presence of AKBA at 0, 50, 75 and 100 µM. Left. Western blot development with anti-5-LOX body antibody. Right. Western blot development with anti-5-LOX tail antibody. The westerns indicate the fading cleavage bands with increasing AKBA concentrations.
density analysis demonstrated an increase in the FLAP-ND band in the 5-LOX-FLAP-ND incubations as AKBA concentrations are increased. The FLAP-ND control was loaded at an equivalent level to FLAP-ND in the 5-LOX-FLAP-ND incubations. This data suggests that AKBA may interfere with 5-LOX's ability to interact with FLAP. Therefore, this impairment of 5-LOX to interact with FLAP would eliminate 5-LOX's ability to access substrate from FLAP therefore halting the initiation of LT biosynthesis.

Our results suggest that when AKBA is bound the regio-specificity 5-LOX is compromised, as well as its interaction with FLAP. The latter process may dominate in a cellular context, since the alternate product regio-specificity (12-HPETE) is not observed in the presence of FLAP in the HEK cells. However, when polarized macrophages expressing both 5-LOX and FLAP are treated with AKBA, both inhibition of LT production and an increase in 12-HPETE are observed (Data from Jana Gerstmeier). A confounding aspect of the macrophage experiments is the presence of 15-HPETE in the M1 macrophage extracts. However, the LC-MS-MS does not distinguish between 15-S-HPETE and 15-R-HPETE which is produced by Cyclooxygenase.

**Impairment of Substrate Access from FLAP and Change in Product Specificity**

We have demonstrated that the binding of AKBA to 5-LOX inhibits 5-LOX's access to its substrate, AA, from FLAP. FLAP is an AA-binding protein that would sequester nearby AA that has been cleaved out of the nuclear membrane by phospholipase A2 (PLA₂). Therefore, this data suggests that a 5-LOX and FLAP interaction could be necessary for the initiation of LT biosynthesis and that the binding of AKBA to 5-LOX disrupts this interaction. In the absence of FLAP, 5-LOX still has access to the substrate directly from the nuclear membrane and therefore has the ability to produce product. However, the binding of AKBA to 5-LOX may induce a conformational change that affects the organization of the catalytic components in a way that favors the production of 12-HPETE over 5-HPETE.
Upstream of the amino terminal end of helix-α2 is R165, which makes a cation-pi interaction with W102 of the membrane-binding domain. This highly conserved cation-pi interaction, which is suggested to serve as a communication link between the membrane binding and catalytic domains (130), is disrupted in the AKBA-5-LOX structure. Structural differences near helix-α2 could affect active site access and the enzyme’s interaction with FLAP, which presumably must “deliver” the substrate to an open cavity. Moreover, it can impact the regio-specificity of the enzyme by modulating the flexibility of this segment that must undergo a conformational change for substrate to be positioned at the catalytic machinery. The successive reactions catalyzed by 5-LOX (conversion of the substrate to the 5-hydroperoxy derivative and transformation of the intermediate to LTA₄) are both initiated by H abstraction from a pentadiene: in the first 5-LOX attacks C7, and the second C10(107). The 12-HPETE isomer would be generated if 5-LOX were to attack C10 first. The presence of AKBA may simply favor a conformation of the active site in which C10 is positioned between the active site iron and the O₂ pocket that lie at the base of the “U-shaped” site.

The AKBA allosteric site establishes a template for the design of inhibitors that function by restricting 5-LOX from accessing its full repertoire of conformational states. Moreover, as the presence of FLAP is essential for the full inhibitory effect of AKBA, and only 5-LOX utilizes the helper protein, exploitation of the AKBA binding groove may be a potent strategy for isoform-specific inhibition.
V. Preliminary Studies of the Inhibition of 5-LOX by J147 and CNB-001

Alzheimer’s disease (AD) is a neurodegenerative disease in which the accumulation of amyloid-like proteins in neurons cause nerve cell death. This is primarily exhibited in older adults as the increase in accumulation of the proteins is associated with increased age. The buildup of amyloid-like proteins induces the inflammatory response and the increase in the protein amyloid beta (Aβ) has been demonstrated to cause an increase in eicosanoids, including the pro-inflammatory leukotrienes (LTs) (131). The initiation of the inflammatory response by amyloid-like proteins may be a contributor to the progression of AD by enhancing nerve cell death. Thus, targeting 5-lipoxygenase (5-LOX), the initiator of LT biosynthesis could have a positive effect on combating the progression of AD.

What Are J147 and CNB-001?

Both J147 and CNB-001 are compounds that have demonstrated promise for treatment of Alzheimer’s disease (AD). CNB-001 and J147 are compounds that have been derived from the natural product curcumin, which has demonstrated positive results in the fight against AD (132). CNB-001, which has a similar structure to other 5-LOX inhibitors, is a pyrazole derivative of curcumin and J147 is derived from the CNB-001 compound. AD mouse studies with CNB-001 demonstrated better neuroprotective properties over curcumin, while J147 proved to have even more effective neuroprotection and exhibited effects of an increase in brain derived neurotropic factor (BDNF), as well as a reduction in inflammation and oxidative stress (132). Both of these compounds have been shown to inhibit 5-LOX. Stress on the endoplasmic reticulum (ER) has been suggested to play a crucial role in AD, and 5-LOX may have a part in that stress accumulation (133). Inhibition of 5-LOX by CNB-001 has been demonstrated to decrease amyloid beta (Aβ) formation and cell death in MC65 cells(131). However, the mechanism of action between 5-LOX and AD is not yet understood. The initial trigger of the inflammatory response by amyloid-like proteins may enhance the early cell damage caused by the aggregation of these proteins.

Results

HEK293 cells transfected with 5-LOX were analyzed for 5-LOX activity in the presence of either J147 or CNB-001. 5-LOX products were analyzed by HPLC analysis. 5-LOX activity was measured
both in the absence and presence of stimulation by calcium ionophore A23187. CNB-001 displayed complete 5-LOX inhibition at 50µM both in the absence and presence of calcium ionophore stimulation (Figure 33). J147 at this same concentration demonstrated complete inhibition of 5-LOX in the absence of calcium ionophore stimulation but only partial inhibition when 5-LOX expressing HEK cells were stimulated with calcium ionophore.

Immuno-fluorescence (IF) microscopy was employed to characterize the effect of either J147 or CNB-001 on the localization of 5-LOX to the nuclear membrane and colocalization of 5-LOX and FLAP. HEK cells were incubated at 50 µM of either J147 or CNB-001. Rabbit anti-5-LOX and goat anti-FLAP antibodies were used to detect each protein and secondary antibodies donkey anti-

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**Figure 34.** Immuno-fluorescence microscopy of 5-LOX expressing HEK293 cells incubated with J147 or CNB-001. DAPI staining depicts the nucleus. Green (Alexfluor 488 detected with GFP filter) represents 5-LOX.

**Figure 35.** Immuno-fluorescence microscopy of 5-LOX-FLAP expressing HEK293 cells incubated with J147 and CNB-001. DAPI depicts the nucleus. Green (GFP filter) represents 5-LOX and red (Cy5 filter) represents FLAP.
rabbit Alexfluor 488 and donkey anti-goat Alexfluor 647 were observed by IF microscopy. In 5-LOX only expressing cells which were stimulated with calcium ionophore A23187, incubations with J147 and CNB-001 did not disrupt 5-LOX localization to the nuclear membrane (Figure. 34).

In HEK cells expressing both 5-LOX and FLAP, 5-LOX localization to the nuclear membrane and colocalization with FLAP were not disrupted with J147 but incubation with CNB-001 did display a decrease colocalization of 5-LOX and FLAP at the nuclear membrane (Figure. 35) as depicted in figure 36. The colocalization scatter plots display Mander’s coefficient and Pearson’s correlation of the green and red fluorescence representative of 5-LOX and FLAP, respectively (Figure. 36) Channel 1 represents red (FLAP) and channel 2 represents green (5-LOX). The DMSO control and J147 incubations display the colocalization of green and red presented as yellow while the CNB-001 incubation displays a dispersed green fluorescence that lays outside the vicinity of the red fluorescence.

**Impairment of Colocalization between 5-LOX and FLAP**

While unstimulated HEK cells expressing 5-LOX are inhibited by J147, J147 does not fully inhibit the cells when stimulated with calcium ionophore A23187. This may suggest that at 50 µM the concentration of J147 is not high enough to fully inhibit the enzyme. However, 50 µM of CNB-001 fully inhibits 5-LOX product formation in both stimulated and unstimulated cells. How this relates to the fact that CNB-001 may interfere with 5-LOX-FLAP colocalization is not clear at this point. IF analysis displayed a sustained colocalization between 5-LOX and FLAP when HEK cells were incubated with J147 while HEK cells incubated with CNB-001 displayed a reduced colocalization between the two proteins.

Previous studies have demonstrated J147 to be a more potent suppressor of AD(132) than CNB-001, however CNB-001 appears to be a more effective inhibitor of 5-LOX than J147. This could suggest that J147 has other roles in suppressing AD besides inhibiting 5-LOX that have made it a more effective candidate for treatment of AD. Both J147 and CNB-001 have been shown to be effective drug candidates for treatment of AD. It is clear that 5-LOX may have a significant part in the progression of AD, however more information needs to be obtained on the exact function of 5-LOX and thus LT biosynthesis in the initiation and/or progression of AD and the mechanism of action in which J147 and CNB-001 function to decrease the accumulation of amyloid proteins, and therefore significantly reduce symptoms of AD.
VI. Material and Methods

5-LOX Purification: His-tagged WT-5-LOX was expressed in Rosetta cells with a pET14B vector. Bacteria were cultured at 37°C and 220 rpm for 4 hours and then at 20°C for an additional 27 hours. Cells expressing WT-5-LOX were lysed by sonication and the lysate was clarified by centrifugation at 36,000 x g for 30 minutes. To the supernatant was added ammonium sulfate to 50% saturation. The precipitant was pelleted and resuspended in 100 mM Tris, 100 mM NaCl, 5 mM Imidazole, 2 mM tris(2-carboxyethyl)phosphine (TCEP), 10 μM FeSO₄, 10 μg/ml catalase. The resuspended precipitant was applied to a Co²⁺-affinity column. The column was washed with 100 mM Tris, 100 mM NaCl, 2 mM TCEP, 10 μM FeSO₄, 20 mM imidazole and the immobilized enzyme eluted with 100 mM Tris, 100 mM NaCl, 2 mM TCEP, 10 μM FeSO₄, 10 μg/ml catalase, 200 mM imidazole. After concentration of the eluant, protein concentration was determined with a Nanodrop spectrophotometer. Protein purity was confirmed by SDS-PAGE. Purified protein was frozen dropwise and stored in liquid N₂.

5-LOX-TKM Purification: Bacterial cell cultures prepared as above were lysed by French press. After clarification of the lysate by centrifugation at 36,000 x g, the supernatant was applied to a Co²⁺-affinity column. The column was washed with 100 mM Tris, 500 mM NaCl, 20 mM imidazole. The immobilized enzyme was eluted with 100 mM Tris, 500 mM NaCl, 200 mM imidazole. After concentration of the eluant, protein concentration was determined with a Nanodrop spectrophotometer. Protein purity was confirmed by SDS-PAGE. Purified protein was frozen dropwise and stored in liquid N₂.

Expression of 5-LOX and FLAP in HEK293 cells: HEK293 cells were transfected with WT-5-LOX, TKM-5-LOX, and FLAP using the pcDNA3.1 vector for stable protein expression as described by Gerstmeier et al (32). Cells were cultured at 37°C, 5% CO₂ in Dubelco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Atlanta Biologicals), in the presence of selection antibiotic (G418 for 5-LOX; and hygromycin for FLAP (both from Sigma)). Successful protein expression was confirmed by Western blotting.

Proteolytic Cleavage: Purified enzyme. Purified WT- and TKM-5-LOX were diluted to 9.8 μM in 100 mM Tris, 100 mM NaCl. The purified protein solutions were incubated with or without 2.5 μM CaCl₂ for 5 minutes. The solutions were then incubated with or without 5 μM chymotrypsin (2:1 protein to protease ratio) for 1 minute on ice. Samples were run on an SDS-PAGE gel and transferred to a PVDF membrane with a Bio-Rad Trans-Blot system for Western development. The primary antibodies were rabbit anti-5-LOX “Body” (amino acids 130-149) and rabbit anti-5-LOX “Tail” (12 amino acids from the C-terminus). The secondary antibody utilized was goat anti-rabbit AlexFlour647. Western blots were imaged with a Typhoon 9410 imager using channel 670 30 BP.

Cell lysates. HEK293 cells expressing WT- or TKM-5-LOX were collected and resuspended at 1 x 10⁸ cells/ml in phosphate-buffered saline (PBS) plus 0.1% glucose. Cells were incubated with or without 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes in a 37°C water bath. The cells were spun down and resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2% Triton-X100), before being incubated with 100 nM chymotrypsin for 1 minute on ice. Sample protein concentrations were determined with a BCA protein assay kit (Pierce). Equivalent amounts of protein were loaded onto 10% SDS-PAGE gels. After blotting onto PVDF membranes. The resulting Western blots were developed as described above.
Proteolytic Cleavage in Presence of AKBA: *Purified enzyme.* Purified WT-5-LOX was diluted to 9.8 μM in 100 mM Tris, 100 mM NaCl. The purified protein solutions were incubated with or without 2.5 μM CaCl₂ for 5 minutes followed by incubation with AKBA at 0, 25, 50, 75 and 100 μM concentrations for 10 minutes. The solutions were then treated as described above.

**Cell lysates.** HEK293 cells expressing WT-5-LOX with or without FLAP were collected and resuspended at 1 x 10⁶ cells/ml in phosphate-buffered saline (PBS) plus 0.1% glucose. Cells were incubated with or without 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes at 37°C water bath followed by a 10 minute incubation with AKBA at 0, 25, 50, 75 and 100 μM concentrations. Then the cells were treated as described above.

5-LOX HPLC Product Analysis of HEK293 cells: HEK293 cells expressing WT- or TKM-5-LOX with or without FLAP were collected and resuspended at 1 x 10⁶ cells/ml PBS plus 0.1% glucose. Each sample was incubated with or without 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes at 37°C then incubated for 10 minutes with 3 μM AA (Cayman). The reaction was stopped with the addition of 1 ml (1 volume) of methanol. Prior to solid phase extraction with C18 cartridges (UCT CLEAN-UP C18 CEC1811Z), 0.5 ml PBS, 30 μl of 1 M HCl, and 10 μl of 50 ng/μl prostaglandin B₁ (Cayman, PGB₁) were added. The products were eluted with methanol. The methanol was evaporated off under N₂ gas and the residue was resuspended in 60% acetonitrile, 0.1% formic acid, the HPLC mobile phase. Samples were treated with triphenylphosphine as a reducing agent prior to HPLC analysis. Isocratic reverse-phase HPLC was performed with a Supelco Discovery HSC18 column monitored at 235 and 270 nm. Peaks for 5-HETE (235nm), leukotrienes and PGB₁ (270 nm) were integrated. Activity analysis for HEK cells expressing TKM-5-LOX and FLAP with only endogenous AA pools was performed in the same fashion above except without added AA and 5 incubations were pooled for solid phase extraction of products.

5-LOX Product Analysis by HPLC in the Presence of AKBA: *Purified enzyme.* Purified WT-5-LOX was diluted to 9.8 μM in 100 mM Tris, 100 mM NaCl. The purified protein solutions were incubated with or without 2.5 μM CaCl₂ for 5 minutes followed by incubation with AKBA at 0, 25, 50, 75 and 100 μM concentrations for 10 minutes. The solutions were then treated as described above.

**Cell Lysates.** HEK293 cells expressing WT-5-LOX with or without FLAP were collected and resuspended at 1 x 10⁶ cells/ml PBS plus 0.1% glucose. Each sample was incubated with 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes at 37°C, followed by incubation with AKBA at 0, 25, 50, 75, and 100 μM concentrations before being treated as described above.

15-LOX-2 Product Analysis in the presence of AKBA: HEK293 cells expressing WT-15-LOX-2 were collected and resuspended at 1 x 10⁶ cells/ml PBS plus 0.1% glucose. Each sample was incubated with 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes at 37°C, followed by incubation with AKBA at 0, 25, 50, 75, and 100 μM concentrations before being treated as described above.

5-LOX Product Analysis in HEK293 Cells in the Presence of J147 and CNB-001: HEK293 cells expressing WT-5-LOX were collected and resuspended at 1 x 10⁶ cells/ml PBS plus 0.1% glucose. Each sample was incubated with 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes at 37°C, followed by incubation with J147 or CNB-001 at 50 μM concentration before being treated as described above.
Immunofluorescence Imaging of 5-LOX and FLAP in HEK293 Cells: HEK293 cells expressing WT- or TKM-5-LOX with or without FLAP were seeded out at 200 cells/ml in each well of an Ibdi 12-well slide. After 24-48 hours at 37°C 5% CO₂, 2.5 μM Ca²⁺-ionophore A23187 was added to stimulate the cells, which were then incubated for 5 minutes at 37°C 5% CO₂. The cells were fixed with 4% paraformaldehyde then washed 3 times with PBS. The cells were then incubated with a 50 mM NH₄Cl solution, followed by a wash cycle and subsequently blocked for an hour with 10% donkey serum, 0.1% Tween-20 in PBS. The samples were then incubated overnight at 4°C with a primary antibody solution consisting of either rabbit anti-5-LOX “Body” +/- goat anti-FLAP or rabbit anti-5-LOX “Tail” +/- goat anti-FLAP in 0.1% Tween-20 PBS solution. After a wash cycle with 0.1% Tween-20 PBS the cells were incubated with a secondary antibody solution consisting of donkey anti-rabbit AlexaFluor488 +/- donkey anti-goat AlexaFluor647 in a 0.1% Tween-20 PBS solution for 20 minutes. After another wash cycle, the cells were incubated with 2 μg/μl DAPI solution for 3 minutes followed by another wash cycle. Invitrogen Prolong Gold antifade reagent and a coverslip were then added to the slide. The slide was imaged with a Leica DM6B upright microscope using DAPI, GFP (5-LOX), and Cy5 (FLAP) filters.

Immunofluorescence Imaging of 5-LOX and FLAP in HEK293 Cells in the Presence of AKBA: HEK293 cells expressing WT-5-LOX with or without FLAP were seeded out at 200 cells/ml in each well of an Ibdi 12-well slide. After 24-48 hours at 37°C 5% CO₂, 2.5 μM Ca²⁺-ionophore A23187 was added to stimulate the cells, which were then incubated for 5 minutes at 37°C 5% CO₂. AKBA was then incubated at 0, 25, 50, 75, and 100 μM concentrations for 10 minutes at 37°C 5% CO₂. Then cells were prepared for immunofluorescence imaging as stated above.

Immunofluorescence Imaging of 5-LOX and FLAP in HEK293 Cells in the Presence of J147 and CNB-001: HEK293 cells expressing WT-5-LOX with or without FLAP were seeded out at 200 cells/ml in each well of an Ibdi 12-well slide. After 24-48 hours at 37°C 5% CO₂, 2.5 μM Ca²⁺-ionophore A23187 was added to stimulate the cells, which were then incubated for 5 minutes at 37°C 5% CO₂. J147 or CNB-001 were then incubated at 50μM concentrations for 10 minutes at 37°C 5% CO₂. Then cells were treated as stated above.

HEK Cell Protein Time-course in the Absence of Protein Synthesis: HEK cells expressing WT- or TKM-5-LOX with or without FLAP were collected and resuspended at 1 x 10⁶ cells/ml PBS plus 0.1% glucose. The cells were incubated with or without 2.5 μM Ca²⁺-ionophore A23187 for 5 minutes and then treated with 200 μM cycloheximide at 37°C and analyzed over eight time points (0, 60, 90, 120, 150, 180, 210, and 240 min). An extra 200 μM cycloheximide was added after 120 minutes to ensure continuous inhibition of protein synthesis. At each time point 2 x 10⁶ cells were treated with lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2% Triton-X100, 1 μM pepstatin, leupeptin and PMSF). Sample protein concentrations were determined with a BCA assay and equal amounts of protein were loaded onto 10% SDS-PAGE gels. The samples were then analyzed by Western blot using rabbit anti-5-LOX “Tail” and goat anti-GAPDH primary antibodies and donkey anti-rabbit AlexaFluor488 and donkey anti-goat AlexaFluor647 secondary antibodies. Western blots were imaged with a Typhoon 9410 imager using channels 526 SP and 670 30 BP. For quantitative analysis of western bands we used ImageJ software.

qPCR HEK 5-LOX mRNA Quantification: RNA Isolation. Samples of 1 x 10² cells were collected for each of the four cell lines, i.e., WT- or TKM-5-LOX with and without FLAP. After spinning down cells and removing the media, the cells were resuspended in Trizol Reagent and incubated for 5 minutes at room temperature. Then, a 50/50 mixture of chloroform and isoamyl alcohol was mixed with the samples and allowed to incubate for 3 minutes at room temperature. After centrifugation,
the upper aqueous phase of the samples was transferred to a fresh tube. Isopropanol was mixed with aqueous phase and incubated for 10 minutes at room temperature. The samples were again centrifuged and the RNA pellet was washed twice with 75% ethanol and then let to air dry. The RNA pellets were then dissolved in diethylpyrocarbonate (DEPC)-treated water before undergoing DNase treatment where the samples were incubated with DNase I at 37°C for 30 min then with the addition of 10 mM EDTA incubated at 70°C for 10 min. Next, the samples were incubated with 300 mM sodium acetate and 2.5 volumes of ethanol at -80°C for 15 min before being centrifugation at 4°C for 10 min. The RNA pellets were dissolved in DEPC-treated water and concentration readings were taken by Nanodrop.

**cDNA Synthesis and Gel Analysis:** The isolated RNA (10 μg) served as a template for cDNA synthesis using the Genescript reverse transcriptase kit (Promega). Forward and reverse oligos for 5-LOXs and the GAPDH control were made by Integrated DNA Technologies (IDT) (Fig. S5). Samples were then treated with RNaseH before undergoing PCR. The PCR samples were run on an Agarose gel and analyzed visually. For quantitative analysis we used ImageJ software.

**FLAP Purification:** His-tagged FLAP was expressed in C43 (DE3) cells using the pET21b vector. The cells were cultured at 37°C, 200rpm for 4 hours, induced with IPTG, and incubated an additional 24 hours at 15°C. After cell lysis by sonication, the cell debris was cleared and 0.1% dodecylmaltoside (DDM) was added and the sample was extracted at 4°C overnight. The lysate was subsequently applied to a Co²⁺-affinity column. The column was washed with 20mM Tris, 300 mM KCl, 50mM Imidazole, 20% glycerol and 0.1%DDM and the immobilized protein eluted with 20mM Tris, 300mM KCl, 250mM Imidazole, 20% glycerol and 0.1%DDM. After concentration of eluant, protein concentration was determined with a Nanodrop spectrophotometer. Protein purity was confirmed by SDS-PAGE. Purified protein was frozen dropwise and stored in liquid N₂.

**Membrane Scaffolding Protein (MSP) Purification:** His-tagged MSP1E3D1 was expressed in Rosetta 2 (DE3) cells using the pET28 vector. Cells were cultured at 37.5°C 250 rpm for 4-6 hours. Cells were induced with 500 mM IPTG and let to culture for an hour at 37.5°C before lowering the temperature to 28°C for an additional 3 hours. After lysing cells by sonication and clarifying the lysate, the lysate was applied to a Co²⁺-affinity column. The column was washed with four different buffers each containing 40mM Tris-HCl 300mM NaCl at pH 8.0: Buffer 1 (+1% Triton), Buffer 2 (+50mM cholate), Buffer 3 (no addition), Buffer 4 (+20mM Imidazole). The protein was eluted with 40mM Tris-HCl 300mM NaCl 300mM Imidazole. After concentrating, the protein A₂₈₀ was read on a Nanodrop. Protein was frozen in liquid N₂ and stored at -80°C.

**Empty-Nanodisc Construction:** A phospholipid mixture of 75% (POPC), 25% (POPS), and 2% (SAPC) was suspended in 20mM Tris, 150 mM NaCl, 0.5 mM EDTA and 100 mM cholate. Purified MSP was added to a phospholipid mixture at a 140:2 PL:MSP ratio. Additional cholate was added as necessary to maintain the cholate concentration at 25 mM or more. The mixture with a total volume of 100μl was incubated for one hour on a nutator at 4°C. Then ~5 Bio-Beads were added to mixture and rotated overnight at 4°C. The solution was then run through Bio-Beads to adsorb all the remaining cholate. The Nanodiscs were purified by Co²⁺ affinity chromatography. After concentration, the A₂₈₀ was read on a Nanodrop. The nanodisc solution was then stored at 4°C.

**FLAP-Nanodisc Construction:** A phospholipid mixture of 75% (POPC), 25% (POPS), and 2% (SAPC) was suspended in 20mM Tris, 150mM NaCl, 0.5mM EDTA and 100mM cholate. Purified MSP was treated with tobacco etch virus (TEV) protease to remove Histidine tag and termed clipped MSP (cMSP). Purified cMSP and FLAP were added to phospholipid mixture at a 140:2:2 PL:MSP:FLAP ratio. Additional cholate was added as necessary to maintain the cholate
concentration at 25mM or more. The mixture with a total volume of 200μl was incubated for one hour on a nutator at 4°C. Then ~5 Bio-Beads were added to mixture and rotated overnight at 4°C. The solution was then run through Bio-Beads to adsorb all the remaining cholate. The FLAP-ND were purified by Histidine affinity chromatography. After concentration, the A$_{280}$ was read on a Nanodrop. The nanodisc solution was then stored at 4°C.

5-LOX-FLAP/EMPTY-Nanodisc Assembly: 5-LOX with either Empty or FLAP nanodiscs were incubated together at a 1:1 ratio for 30min at 4°C. The 5-LOX-FLAP/Empty-ND complex was purified by size exclusion chromatography (SEC) with a running buffer of 20mM Tris 150mM NaCl and 0.5mM EDTA. The peak fractions were concentrated with 30K Amicon filters and run on a gel which were stained with Sypro Ruby as well as analyzed by Western blot for detection of both 5-LOX and FLAP.

Negative Stain Transmission Electron Microscopy (TEM) Imaging: A 300 square mesh copper grid was glow discharged for 30 seconds before 4μl of sample of either Empty-NDs, FLAP-NDs, 5-LOX-Empty-NDs or 5-LOX-FLAP-NDs was applied for 1 minute. After blotting with filter paper, the grid was incubated on a drop of 2% uranyl acetate for 3min, then blotted and left to dry. Imaging was done on a JEOL 1400 TEM operating with LaB6 filament at 120kV. Images were captured with a 2K x2K CCD camera.

Cryo-TEM Imaging of Empty-Nanodiscs: Quantifoil R2/4 400 mesh copper holey grids were glow discharged for 30 seconds before 5μl of Empty-ND sample was applied. Using a Gatan Cryo Plunge, grids were mechanically blotted on both sides for 2 seconds before being plunged in to liquid ethane and vitrified. The sample was imaged on the instrument stated above with the minimal dose system (MDS) and kept under cryo conditions with a Gatan cryo-holder.

Immuno-gold and Negative Stain TEM Imaging: Either FLAP-NDs or 5-LOX-FLAP-NDs were applied to glow discharged 300 square mesh copper grids for 3min. After blotting with filter paper, the grid was placed on a drop of 4% PFA for 10min. Then the grid went through a 3 drop wash cycle at 5min/drop with PBS, a 50mM NH$_4$Cl incubation for 10min, and lastly another wash cycle. Following a blocking solution incubation of 1hr, the grid was then incubated in either rabbit anti-5-LOX or goat anti-FLAP primary antibody solution for 1hr. After a wash cycle, the grid was incubated for an additional hour in either donkey anti-rabbit or donkey anti-goat 10nm immuno-gold secondary antibody solution, followed by a wash cycle. The grid was then stained with 2% uranyl acetate for 2min, blotted and let to dry. Images were taken on a JEOL 1400 at 120kV.

Native Gel Analysis of 5-LOXWT-FLAP-Nanodisc Complex: Purified WT-5-LOX, FLAP, Empty-NDs, FLAP-NDs, 5-LOX-Empty-NDs, and 5-LOX-FLAP-NDs were run on a clear native gel. 5-LOX and FLAP-ND were incubated at either a 0.25:1, 0.5:1, 1:1, or 2:1 5-LOX:FLAP-ND ratio with 100nM CaCl$_2$ for 5 minutes on a nutator at 4°C. Each sample was loaded and run on a native gel at 4°C for 4-5 hours. Gels were fixed with 50% methanol 7% acetic acid solution before being stained with Sypro ruby overnight. Western blot of gel was developed with a goat anti-FLAP primary antibody and a donkey anti-goat AlexFluor 647 secondary antibody. Gels and westerns were imaged with Typhoon 9410 imager using channel 610 and channel 670 30 BP, respectively.

Native Gel Analysis of 5-LOXWT-FLAP-Nanodisc Complex in Presence of AKBA: Purified WT-5-LOX and FLAP-Nanodiscs were incubated at a 1:1 ratio for 5 minutes before being incubated with
AKBA at 0, 25, 50, 75, and 100 μM concentrations for 10 minutes on the nutator at 4°C. Native gel was run as stated above.
Conclusion

Although there has been a significant amount of research done on the leukotriene biosynthesis pathway, more information is needed to fully understand its regulation and the balance of this pathway with other arachidonic acid derived lipid mediator pathways. My research has contributed insight into the regulation of 5-LOX and FLAP as the initiators of LT biosynthesis.

Analyzing the unique triple lysine motif (KKK<sup>653-655</sup>) in 5-LOX gave us information into why 5-LOX would require the helper protein, FLAP. My data suggests that this motif acts as a modulator of 5-LOX activity for the effective balance of LT biosynthesis. Product analysis done with HEK293 cells demonstrated that WT-5-LOX stimulated to the nuclear membrane in the absence of FLAP displayed a weak production of 5-HETE and LTs. However, if this motif is mutated to a more stable sequence (ENL) a significant ~20-fold increase in product formation was observed. Moreover, when either wildtype or the mutant was expressed with FLAP, product formation increased 3-fold for WT-5-LOX but the mutant activity was reduced to wildtype levels. This data displays a Goldilocks story where 5-LOX with the KKK motif produces too little LTs for an effective inflammatory response and the ENL substitution of this motif produces too much, therefore causing a detrimental response. The WT-enzyme with FLAP produces just the right amount of products for effective LT biosynthesis. Moreover, the elevated activity of the mutant enzyme was abolished when product analysis was examined in cell homogenates, where the cell membranes are broken. Therefore not only does FLAP play an important regulatory role in LT biosynthesis, but the 5-LOX triple lysine motif may require the membrane for its role in modulating 5-LOX activity. This data along with the observation of turn-over dependent inactivation through cellular protein sustainability analysis demonstrates this motif to be an intricate and finely-tuned control mechanism to regulate LT biosynthesis in conjunction with FLAP. The next step in analyzing the triple lysine motif is addressing the role of the nuclear membrane in the function of this motif to better understand exactly how the KKK motif modulates 5-LOX activity.

The observation of allosteric inhibition of 5-LOX by the natural product AKBA and its effect on the 5-LOX-FLAP interaction has provided new insights into a unique inhibitor development. The x-ray crystal structure of Stable-5-LOX with AKBA bound solved by Dr. Nathaniel Gilbert defined an allosteric binding site for AKBA between 5-LOX’s membrane binding domain and its catalytic domain. Product analysis of 5-LOX in HEK293 cells in the presence of AKBA demonstrated a change in product specificity by 5-LOX-only expressing cells. The 5-LOX expressing cells produced 12-HETE over 5-HETE with increasing concentrations of AKBA. However, with cells expressing both 5-LOX and FLAP, LT formation was diminished and 5-HETE cut in half at 25µM AKBA incubations. All product production was lost in incubations with higher concentrations of AKBA. Immuno-fluorescence microscopy of 5-LOX and FLAP in HEK cells displayed a decrease in colocalization of the two proteins at the nuclear membrane. This data suggests that AKBA may disrupt the interaction between 5-LOX and FLAP, therefore preventing 5-LOX’s access to substrate from FLAP. Native gel analysis of an in vitro 5-LOX-FLAP-ND complex supports this further by demonstrating a decreased association between 5-LOX and FLAP-ND in the presence of AKBA. This data demonstrates that the binding of AKBA to 5-LOX may induce a small conformational change in 5-LOX that changes the product specificity of the active site as well as prevents substrate acquisition from FLAP. This allosteric inhibition may display the ability of AKBA to inhibit pro-inflammatory responses (LTs) while promoting anti-inflammatory responses (5-HPETE/12-HPETE).
The next steps in analyzing this inhibitor as an effective anti-inflammatory compound is to address how AKBA affects the balance of pro and anti-inflammatory LOX mediated pathways, as well as creating a mimic of AKBA that could effectively be transported into cells.

Reconstructing the 5-LOX-FLAP interaction in vitro with the use of nanodiscs allowed us the opportunity to analyze the structural and functional relationship between 5-LOX and FLAP. Previous studies have strongly suggested an interaction between 5-LOX and FLAP, but it has never been proven. Moreover, an interaction between solubilized 5-LOX and detergent solubilized FLAP has never been observed in vitro. The membrane appears to play a significant role in the 5-LOX-FLAP interaction. The incorporation of FLAP into nanodiscs allowed for a favorable interaction between 5-LOX and FLAP-NDs. Nanodiscs were constructed with lipids that mimicked the lipid make-up of nuclear membrane. Empty-NDs, FLAP-NDs, 5-LOX-Empty-NDs, and 5-LOX-FLAP-NDs were analyzed by single-particle TEM. TEM imaging of FLAP-NDs revealed FLAP as a dark spot inside the center of the ND structure and FLAP was confirmed to be incorporated by immuno-gold labeling of FLAP. TEM imaging of 5-LOX-FLAP-NDs revealed a side view of the complex which displayed two copies of 5-LOX sitting on top a FLAP-ND. Although optimization of complex imaging is needed, current micrographs of the 5-LOX-FLAP-ND complex can be seen coming together. The 5-LOX enzymes demonstrated in the micrograph were measured at 8.9nm which is comparable to the shape and length of the 5-LOX crystal structure. This observation is strengthened by the fact that when 5-LOX-Empty-NDs are imaged by TEM this association between 5-LOX and the nanodiscs is not observed. Therefore, the clustering of 5-LOX and FLAP-NDs is due to the attraction between 5-LOX and FLAP. Furthermore, clear native gel and western blot analysis demonstrated an affinity of 5-LOX for FLAP-NDs by a display of a significant migration change by FLAP-NDs when in the presence of 5-LOX. Preliminary HPLC product analysis of 5-LOX-Empty-NDs and 5-LOX-FLAP-NDs demonstrated substrate acquisition by 5-LOX from either the bilayer of Empty-NDs or FLAP. Although only the intermediate 5-LOX product was detected, further analysis by LC/MS/MS is being done to detect LT products. The next step in studying the 5-LOX-FLAP-NDs interaction is single-particle cryo-EM imaging of the complex and obtaining data sufficient for three-dimensional reconstruction of the 5-LOX-FLAP-ND structure. The overall complex structure with the known individual protein x-ray crystal structures docked inside could provide insight into the domains of 5-LOX that interact with FLAP and/or the membrane. This information would offer a better understanding of the mechanism of the initiation of LT biosynthesis.
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Appendix A: Proof of Copyright

(Figure #1)


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(Figure #2)

Protein Crystal

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Chapter 1  Chapter 2  Chapter 3  Chapter 4  Chapter 5  Chapter 6  Chapter 7  Chapter 8  Chapter 9  Chapter 10  Chapter 11  Chapter 12  Chapter 13  Appendix

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(Figure #4) Electron Microscope Deutsches Museum-Flicker.com
(Figure #5)

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(Figure #8)

Image of JEOL 1400 at the Electron Microscopy Core Facility at the University of Missouri-Columbia

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Appendix B: Supplemental Figures for Chapter III

Supplemental Figure 1. The transformation of AA to LTA₄. Oxygenation of arachidonic acid (top) produces 5-HPETE (middle) which is further transformed to LTA₄ (bottom).

Supplemental Figure 2. Colocalization of 5-LOX and FLAP in HEK 293 cells. Immuno-fluorescence labeling of WT-5-LOX FLAP and TKM-5-LOX FLAP in HEK cells. Colocalization of 5-LOX and FLAP analyzed by ImageJ in development with both “Tail” and “Body” primary antibodies. A. WT-5-LOX FLAP anti-5-LOX “Body” B. WT-5-LOX FLAP anti-5-LOX “Tail” C. TKM-5-LOX FLAP anti-5-LOX “Body” D. TKM-5-LOX FLAP anti-5-LOX “Tail”. All Pearson’s Correlation (Rr) and Mander’s Overlap Correlation (R) numbers represented successful colocalization.
Supplemental Figure 3. qPCR of 5-LOX mRNA from HEK 293 cells. Top, ImageJ band intensity analysis of 5-LOX cDNA relative to GAPDH cDNA in HEK cells. Bottom, Agarose gel of 5-LOX and GAPDH DNA bands. From left to right, HEK 293 untransfected, WT-5-LOX, TKM-5-LOX, WT-5-LOX FLAP and TKM-5-LOX FLAP. Transcript levels do not vary significantly among the samples.

Supplemental Figure 4. TKM-5-LOX FLAP expressing cells show LT formation without the addition of exogenous AA. A. Spectrum consistent with LT metabolites generated in HEK cells expressing TKM-5-LOX and FLAP. B. Overall product levels of TKM-5-LOX FLAP cells stimulated with Ca^{2+}-ionophore A21387.
Supplemental Figure 5. mRNA sequence targets for 5-LOX and GAPDH. A. 5-LOX, 336bp. B. GAPDH, 330bp. Primers are indicated in yellow.
Vita

Erin Elizabeth Schexnaydre was born in 1990 in New Orleans, Louisiana. She graduated from Archbishop Chappelle High School in 2008. Erin received a Bachelor of Science degree in Biochemistry with Honors in 2012 from Louisiana State University (LSU). She worked as an undergraduate researcher in the lab of Dr. Marcia Newcomer from 2009-2012 and defended her undergraduate honors thesis. Erin worked as a research associate with Dr. Newcomer for a year before entering graduate school in 2013. As a graduate student, Erin organized and held a workshop at LSU on the topic of three-dimensional single-particle cryo-electron microscopy (cryo-EM) in order to initiate single-particle cryo-EM at LSU. Erin has worked with Dr. Newcomer for the last five years as a graduate student and is anticipated to graduate this May with a Doctorate of Philosophy in Biochemistry. She plans to continue her career as a scientist in a post-doctoral position with a focus on cryo-electron tomography. Additionally, Erin is an avid triathlete and runner having completed five marathon races and two half-Ironman races. She will be participating in her first full Ironman race this April.