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Understanding the Auto-Regulation of Leukotriene Biosynthesis A Model of Inactivation for 5-Lipoxgenase

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Understanding the Auto-Regulation of Leukotriene Biosynthesis
A Model of Inactivation for 5-Lipoxgenase

By

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Abstract

Understanding the auto-regulation and the initiation of leukotriene biosynthesis starts with the regulation of the enzyme, 5-lipoxygense. Leukotrienes take part in mediating the inflammatory response and they are produced from a reaction catalyzed by 5-lipoxygenase. A complex of proteins, including 5-lipoxygense, is targeted to the nuclear membrane and contributes to the conversion of arachidonic acid to the leukotriene LTA_4. The short-term duration of the inflammatory response supports 5-lipoxygense's fast turn over rate. A model of inactivation was hypothesized in which the carboxy terminal end of 5-lipoxygenase, which binds to the catalytic iron of the protein, is not tightly held in place. Upon inactivation the carboxy terminal end becomes unbound to the catalytic iron and waves freely resembling a tail. The accessibility of the carboxy terminal end was determined by protease cleavage and analyzed with mass spectrometry. Through mass recognition and sequencing, analysis of the protein's accessibility to proteases that can cleave it was examined. Accessibility of the carboxy tail was also determined through western blotting using a carboxy tail specific antibody. Analysis of the model of inactivation for 5-LOX through protease cleavage and specific antibody detection of 5-LOX's carboxy terminal tail when the enzyme is spontaneously inactivated will be addressed in this thesis.

Table of Contents

I. Tables and Figures

II. Introduction

Lipoxygenases are part of a superfamily of dioxygenase enzymes, which contain a non-heme iron at the catalytic center. The catalytic iron binds four ligands; three of which are histidine residues and the fourth being the carboxy-terminus of the protein. 5-Lipoxygenase (5-LOX) is the human form of lipoxygenase while there are also plant and animal forms [2]. LOX is comprised of two domains; one is PLAT (polycystin-1/lipoxygenase/alpha-toxin). A PLAT domain is similar to the Ca^{2+}-dependent domains in several enzymes. This N-terminal domain contains the ability to bind three Ca^{2+} ions. Highly researched 8R-LOX from *Plexaura homomalla* (Caribbean sea whip coral), has demonstrated LOX's Ca^{2+} binding ability [2]. The second domain is the catalytic domain, containing the non-heme iron center. PLAT is the smaller domain with approximately 120 amino acids and comprised of beta-sheets and loops and turns. The catalytic domain is larger and contains mostly alpha-helix regions. LOX contains an arched helix in the catalytic domain, which blocks access to the iron center. The PLAT domain affects the structure of the catalytic domain. For example, the absence of the PLAT domain along with a helix and loop region in 12S-LOX causes the arched helix to be out of position [2].

The C-terminal end bound to the catalytic iron plays an important role in the enzyme's activation and inactivation. The catalytic center is stabilized by the C-terminal end, the three histidine residues, as well as a water molecule, which is positioned on top of the iron atom to keep its oxidized (active) form. Additionally, this 2-His-1 carboxylate triad (H372, H550, and Ile673) contributes to keeping the non-heme iron in its functional position [15]. Lipoxygenases are part of a group of oxidoreductases using its iron to catalyze oxidation-reduction reactions. For example,

arachidonate + O_2 $\rightarrow$ (5Z,8Z,11Z,13E,15S)-15-hydroperoxyicosa-5,8,11,13-tetraenoate

$$\text{arachidonate} + O_2 \xrightarrow{\text{15-lipoxygenase}} \text{(5Z,8R,9E,11Z,14Z)-8-hydroperoxyicosa-5,9,11,14-tetraenoate}$$

$$\text{arachidonate} + O_2 \xrightarrow{\text{8-lipoxygenase}}$$

$$\text{arachidonate} + O_2 \xrightarrow{\text{5-lipoxygenase}} \text{leukotriene } A_4 + H_2$$

The 5-lipoxygenase nucleotide sequence is approximately 82 kb with 14 exons and 13 introns. The N-terminal end is 65kb while the C-terminal end is only about 6kb, which is contrary to their amino acid sequences where the C-terminal sequence is the larger one. Additionally, the 5' end of nucleotide sequence of *5-LOX* contains a GC box instead of the usual "TATA" or "CCAAT" boxes [3]. 5-Lipoxygenase is cell specific; it is found mainly in leukocytes such as neutrophils, eosinophils, monocytes/macrophages, mast cells, B-lymphocytes, dendritic cells, and foam cells, in which it serves its main function in the inflammatory response. The DNA methylation of the 5-LOX nucleotide sequence determines whether or not the 5-LOX enzyme will be expressed [15].

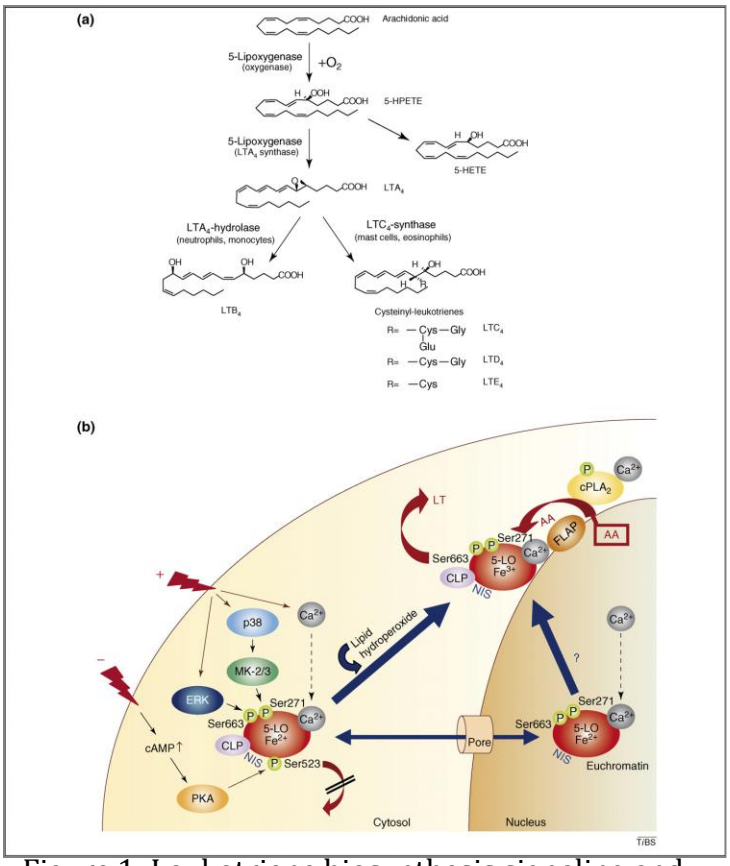

Figure 1: Leukotriene biosynthesis signaling and location of 5-Lipoxygenase in the cell [17]

III. Background

A. Pathway for Leukotriene Biosynthesis

The release of leukotrienes from activated leukocytes promotes the human inflammatory response. These lipid mediators trigger their effect through G-protein coupled receptors (GPCR), BLT1/2, CysLT1/2 and GPR17. The biosynthesis of leukotrienes requires an enzyme-compound complex associated with the nuclear membrane of a cell. This complex can be seen in Figures 1 and 3[17, 18].

Depending on the type of leukocyte, 5-lipoxygenase is either associated with the endoplasmic reticulum (ER) and outer nuclear membrane as in neutrophils or the inner membrane of the nuclear envelope as in alveolar macrophages [1]. 5-Lipoxygenase is targeted to the nuclear membrane by p38, MK, ERK, MAP kinases and/or Ca^{2+} plus DAG. Through Ca^{2+} stimulation, cytosolic phospholipase A_2 is also targeted to the nuclear membrane where it catalyzes a reaction that frees arachidonic acid (AA) from phospholipids as can be seen in figure 3 [18]. ATP was found to increase enzyme activity as well as stabilize the enzyme; however, it is not required for 5-LOX activity. Additionally, even though the binding site for ATP on 5-LOX is unknown, it has been utilized as a purification technique for 5-LOX [15].

5-Lipoxygenase activating protein (FLAP) then transfers AA to 5-lipoxygenase. 5-LOX converts AA to 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE) by inserting molecular oxygen into the C5 of AA [1]. 5-HPETE is transformed further by 5-LOX into 5(S), 6(S)-oxido-7,9,11,15(E,E,Z,Z)-eicosatetraenoic acid (leuktriene A_4, LTA_4), which is an instable epoxide that has several derivatives as shown in Figure 2 and 4 [18].

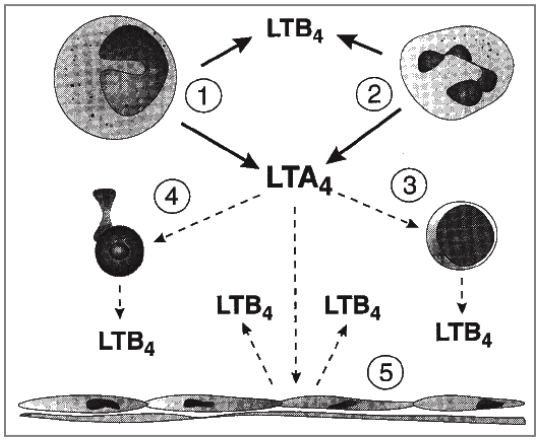

Figure 2: Location differentiation and translocation of the synthesis leukotriene A_4 and its derivatives [18]

One path taken by LTA_4 is its conversion into LTB_4 by LTA_4 hydrolase, which is part of a group of zinc-containing amino peptidases and a soluble enzyme that moves between the nucleus and the cytoplasm [2]. LTA_4 can also be converted into LTC_4 by LTC_4 synthase. LTC_4 synthase is incorporated into the outer nuclear membrane [2].

B. Location of 5-LOX

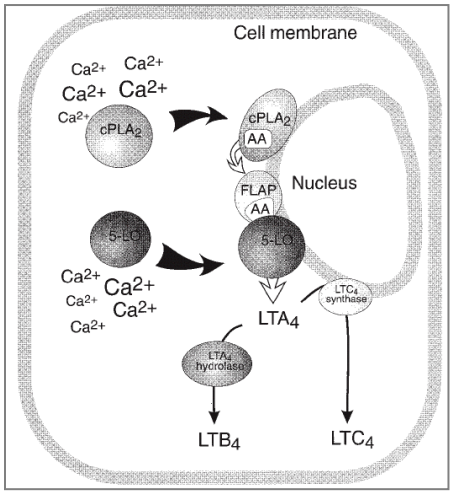

Figure 3: Regulation of the of the activation of 5-Lipoxoygenase [18]

In cells such as, neutrophils, eosinophils and peritoneal macrophages, 5-LOX occupies the cytosol, while in alveolar macrophages, Langerhans cells, and rat basophilic leukemia cells, it occupies nuclear soluble compartments [15]. 5-LOX has nuclear import sequences on its N-terminal end, as well as nuclear export sequences; it is imported into the nucleus upon cell stimulation by glycogen, cytokines, or cell surface adhesion [15]. Additionally, 5-LOX is predicted to have several import sequences indicating a regulation of 5-LOX activity in a particular cell type.

5-LOX's substrate, arachidonatic acid, is additionally a precursor to prostoglandins, isoporstanes, epoxyelcosatrienoic acids and

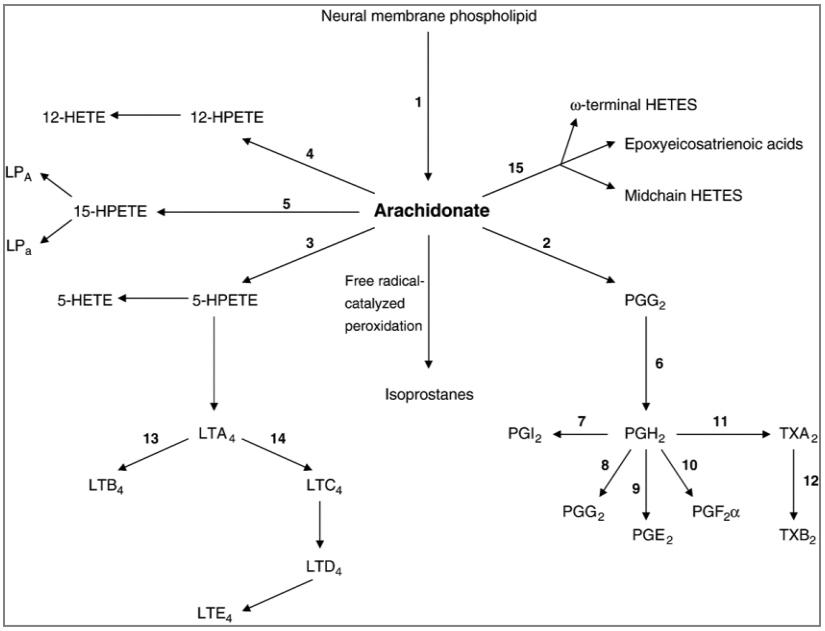

Figure 4: The distribution arachidonatic acid mediation [16].

HETES along with leukotrienes as can be seen in Figure 4 [16].

IV. Theory

The stability of the C-terminus tail is the focus in understanding the auto-regulation of leukotriene production by 5-LOX. 5-LOX's inactivation is key to the enzyme's fast turn over rate. Since the C-terminal end binds to the catalytic center, it is predicted that 5-LOX spontaneously deactivates by the C-terminal tail becoming unbound to the catalytic center, therefore, gesticulating free from the rest of the protein.

A. Molecular Vantage Point

Within the cell 5-LOX spontaneously deactivates and unlike many other proteins, once 5-LOX is denatured it cannot refold into its completely active form. One cause for 5-LOX's fast turn over rate is the catalytic iron's sensitively to O_2, even though other lipoxygenases, such as 8R-LOX, do not display this sensitivity [4]. Additional cell regulatory mechanisms of 5-LOX include regulation by Ca^{2+} due to 5-LOX's beta N-terminal C2 like domain. Calcium stimulation triggers 5-LOX to the nuclear membrane; however, this binding may not necessarily mediate 5-LOX activity, but fluidity of the membrane increases 5-LOX binding to the membrane [15].

B. Chemical Structure Vantage Point

5-LOX has a greater instability in its catalytic center than other LOXs. This difference in instability appears to lie within the C-terminus sequence. In other LOX's the amino acid that allows the C-terminus to position correctly in order to bind the catalytic iron, is Leu. 5-LOX instead has a Lys within the end of the C-terminus [4]. The Lys causes an unfavorable C-terminus conformation of the C-terminus binding to the catalytic center [4]. It is predicted in order to relieve the unfavorable conformation the C-terminus would separate itself from the catalytic iron and enter an inactive state.

In other LOX's, such as 8R-LOX, the lysine-rich (KKK) sequence is an ENL sequence, which is a more stabilizing sequence. When 5-LOX was stabilized by constructing the enzyme with the ENL sequence instead of the KKK sequence, it created a longer half-life and higher melting point for the enzyme [4].

Another stability factor of 5-LOX is the second helix (known as alpha2), which blocks access to the catalytic iron center as well as forms and stabilizes the site with the arched helix. The Stable-5-LOX's (ENL sequence containing enzyme) alpha2 is a significantly shorter with only three turns rather than the six or seven turns in other LOX's such as 8R-LOX and 15-LOX (rabbit lipoxygenase) [4]. In the soluble unstable form of 5-LOX where Lys is positioned instead of Leu, the Lys causes unfavorable cation-π interactions, which lead to 5-LOX's inability to maintain a stable catalytic center [4]

Moreover, these instability factors are reasoned to cause 5-LOX's spontaneous deactivation. Focusing on the catalytic tail that binds to the catalytic center, one hypothesis holds that the tail becomes unbound to relieve the stress of its instability. Therefore, the enzyme becomes inactive, which supports its fast turn over function in the innate immune response. The catalytic domain of 5-LOX and its catalytic center are demonstrated in Figure 5 [17].

The study of 5-LOX's inactivation leads to a further understanding of the auto-regulation of the biosynthesis of leukotrienes. The regulation of leukotriene production corresponds to the modulation of the non-specific inflammatory response. Therefore, further insight into the basis for differing inflammatory response intensities can be concluded. A firm comprehension of the extent of inflammatory response intensities will lead to further progress in differential reasoning in the medical field.

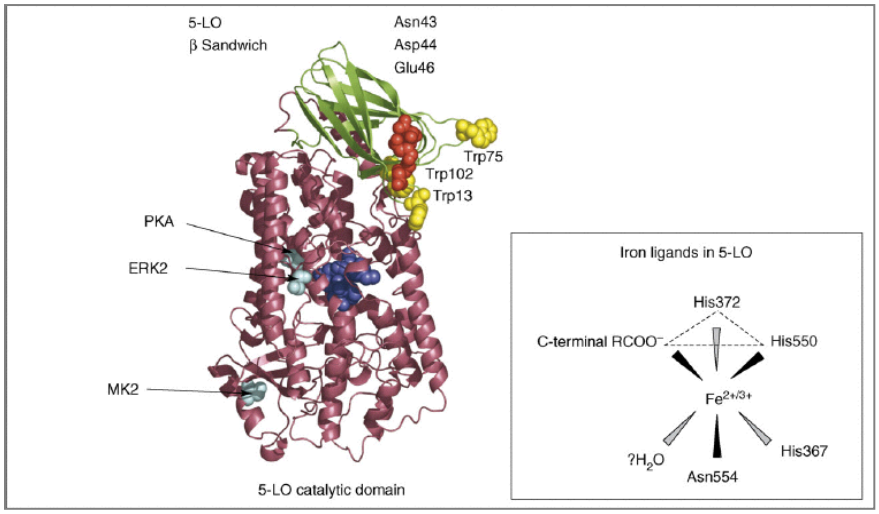

Figure 5: Ribbon diagram of 5-LOX illustrating its catalytic domain and catalytic center as well as some of its nuclear membrane translocation mediators. Structure based on structure of rabbit 15-LOX [17]

V. Materials and Methods

A. SDS-PAGE Gel

Sodium dodecyl sulfate polyacrylamide gel electrophoresis separates protein or protein fragments based on molecular mass. The SDS covers all the protein with an overall negative charge, which allows all the protein to migrate in the same direction. The rate at which the protein migrates to the positive pole is therefore based on molecular mass (size) of the protein or protein fragment. The SDS gel was an important component in observing intact protein and cleavage products of the proteases as well as in protein membrane transfer in later western blot experimental procedures.

B. Protease Cleavage

Trypsin– This serine protease cleaves at the carboxyl end of a protein at Lysine and Arginine amino acids but is stopped if it encounters a Proline amino acid.

Proteinase K– This serine protease cleaves at the peptide bond at the carboxyl end of a protein with aliphatic (Gly, Ala, Val, Leu, Ile) and aromatic (Tyr, Trp, Phe) amino acids. Additionally, this protease retains its activity in SDS.

Chymotrypsin– This enzyme proteolysizes protein by cleaving the peptide amide bond with C-terminal specificity of a Tyrosine, Tryptophan, or Phenylalanine, which have aromatic side chains. Additionally, this protease cleaves the peptide amide bond when there is a Leucine at the carboxyl end, however, this happens at a slower rate than the other cleavage sites.

Carboxypeptidase A– This exopeptidase is a digestive enzyme normally found in pancreatic fluid. It hydrolyzes the peptide bond of a polypeptide chain at the C-terminal end where aromatic and aliphatic amino acids are the side chains.

C. Microcons

Figure 6: Millapore Microcons [19]

Mircocons are centrifugal filters, which contain membranes that filter a particular molecular weight. The membrane desalts and concentrates the experimental sample. The Millipore cellulose membranes YM-10 (10,000 NMWL) and YM-30 (30,000 NMWL) were used in the experimental procedures discussed in this paper. These filters are known to have a high sample recovery rate of 95 percent.

D. Protein Purification Columns

Nickel Column–

The N-terminal end of 5-LOX includes a histidine sequence tag. In a nickel column these histidines will strongly bind to the nickel metal. The column is washed with 20mM concentration of imidazole allowing the protein His tag to bind and for other protein fragments to flow through. The protein is eluted off the column with a higher concentration (200mM) of imidazole. The higher concentration of imidazole competes with the His tag's

affinity for the nickel resin and displaces the bound protein. This allows for purification of the C-terminally cleaved protein and a decrease in the larger signals from protein fragments, which disrupt the signal given by the cleaved protein.

Desalting Column–

The column acted as a buffer exchange column where the protein's Tris-Cl buffer was exchanged with a sodium phosphate buffer. The column was constructed to yield better mass spectrometry analysis even though some protein sample loss is observed in the buffer exchange process.

E. Dialysis

Dialysis was used as another separating technique. Like the microcons it is made up of a cellulose membrane that contains a particular pore size. When placed in double deionized water, the dialysis tubing causes salt to be removed from the protein solution. The diffusion of water through the tubing allows smaller molecules to pass into the larger volume outside the tubing as well as allowing for buffer exchange. Dialysis of the 5-LOX protein allowed for a possible decrease in noise of mass spectrometry results.

F. Nano Drop

This spectrophotometer allows for the analysis of micro-volume samples. The instrument was used to determine the concentration of protein sample by inputting the molecular weight and extinction coefficient values of the protein. It was utilized in the

Figure 7: Micro-Volume Pedestal: Nano Drop Spectroscopy [20]

experiments to determine the protein concentration of samples after protein purification by column and/or microcon procedures.

G. Spectrofluometer

A spectrofluometer is able to detect the fluorescent properties of a compound. The intensity of the fluorescence is reflective of the amount of substrate present that reacts with the fluorescence-producing compound. The concentration of amino acid from protease digestion was observed against known amino acid concentrations.

Fluoraldehyde (OPA)– O-phthalaldehyde is a fluorescent reagent used to detect amino acids. The reagent was utilized for the detection of amino acids after proteolytic cleavage of 5-LOX.

Sigma Plot–Sigma Plot is a scientific data and graph analysis program. The program was used to graph spectrofluometer data results from proteolytic experiments.

Trichloroacetic acid (TCA)– TCA is generally used for the precipitation of macromolecules. TCA was reacted with amino acid solutions and neutralized with sodium hydroxide. The TCA exposed amino acid samples were compared to non-TCA exposed amino acid samples using fluorescence detection.

H. Protease Inhibitors/ Lysate Preparation

1, 10 Phenanthroline monohydrate ($C_{12}H_{10}N_2O$)–

A solution of 60mM 1,10 phenanthroline in ethanol inhibited the carboxypeptidase A used in the proteolytic experiments.

Phenylmethylsulfonyl fluoride (PMSF)– PMSF is a serine protease inhibitor used in preparation of protein lysate.

Peptsatin and Leupeptin- Pepstatin inhibits aspartyl proteases and Leupeptin inhibits cysteine, serine, and threonine proteases. Pepstatin and Leupeptin inhibitors were used in preparation of protein lysates.

Deoxyribonuclease (DNase)– The DNase enzyme catalyzes a hydrolytic cleavage reaction of the DNA backbone.

Bugbuster- Bugbuster is a protein extraction reagent used in lysate preparation. It chemically breaks up the cell wall of E. coli cells and releases proteins that were held inside.

French Press- The French Press is a mechanical technique that lyses E. coli cells and releases the soluble enzymes inside the cell.

I. Ultraviolet-Visible Spectrometer

The UV-Visible spectrometer can detect compounds that have a wavelength in the UV-visible range. Conformation of effective carboxypeptidase A activity was evaluated with this instrument using Hippuryl-L-Phenylalanine.

Hippuryl-L-Phenylalanine– The reaction of this reagent and water is catalyzed by carboxypeptidase A to produce Hippuric acid and L-Phenylalanine. The L-Phenylalanine product has an absorbance at a wavelength of 254 nanometers. The compound was used to test the activity of carboxypeptidase A.

J. Western Blot

Figure 8: Western Blot Apparatus [21]

A western blot is an analytical technique that utilizes antibody specificity to target a particular protein. A protein sample run on a SDS gel is transferred to a PVDF membrane

where antibody binds to the particular target protein with high specificity. Expression of target protein is then observed by use of an antibody chromogenic substrate. The western blot technique contributed to the analysis of the accessibility of the C-terminus tail of 5-LOX.

K. Dynabeads

Dynabeads are magnetic beads used for fast and efficient separation in immunoprecipitation experiments. The antibody of choice (Protein A) can attach to

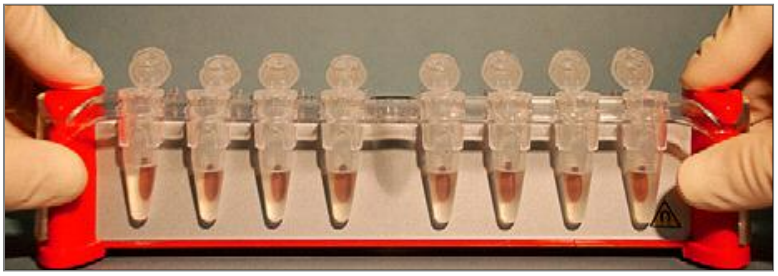

Figure 9: Dynabead magnetic separation

Dynabeads allowing a pull-down of only the antibody specific target. The Dynabead solution is suspended in a 5-LOX C-terminal targeting antibody, therefore, allowing the antibody to bind to protein A on the Dynabeads. After magnetic separation of any non-binding antibody, the Dynabeads-antibody solution is suspended in the protein sample of 5-LOX where the antibody will bind to 5-LOX C-terminus if it is accessible. After magnetic separation of any non-binding proteins, the C-terminal accessible 5-LOXs are then run on a western blot for analysis.

VI. Experimental Analysis

Proteolytic cleavage of OCTA (stable 5-LOX) and Y181A lipoxygenase (5-LOX mutant which replaced a tyrosine for an alanine amino acid at position 181) protein forms demonstrated significant differences.

The excessive cleavage of the Y181A protein by proteinase K compared to cleavage of OCTA by the same protease demonstrated the lower stabilization of Y181A. The trypsin

protease was not as active as proteinase K in cleaving either protein as seen in figures 10 and 11. Trypsin proved to be a more effective protease in observing cleavage of the carboxyl end of 5-lipoxygenase.

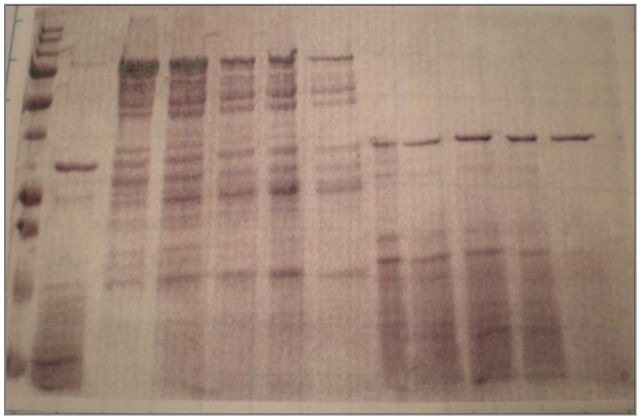

Figure 10: From left: cleavage of Y181A mutant by trypsin(0,15,30,60, and 75min), then cleavage of Y181A with proteinase K.

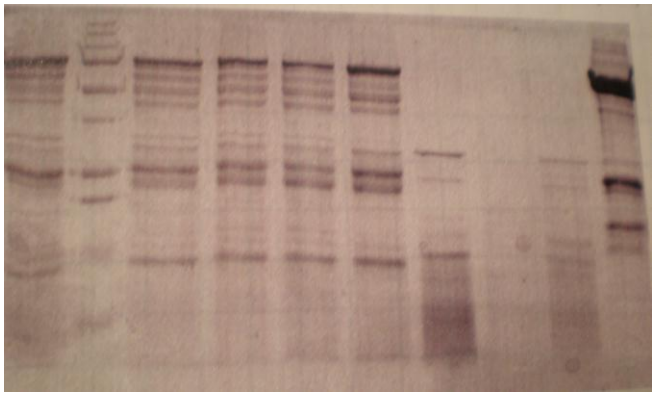

Figure 11: From left: Proteolytic cleavage of 5-LOX with trypsin (0,15,30,60, and 75min), then three wells that include cleavage of 5-LOX by proteinase K and the last well is pure 5-LOX protein.

There is a significant difference in the samples with two microliters of trypsin for the FISH (soluble 5-LOX) lipoxygenase protein over different intervals during an hour. Also, when adding AA at the same concentration the FISH protein demonstrated a larger difference in bands. This evidence demonstrates less exposure of the protein to the protease due to AA interaction. Additionally, the experiment demonstrated the appropriate concentration of trypsin needed to avoid excessive protein fragmentation.

Typsin Protease Experiments

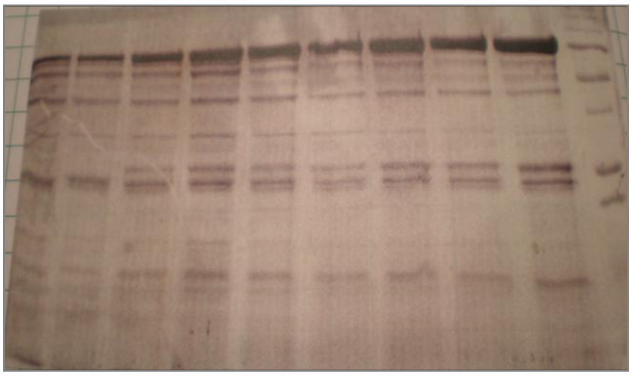

Figure 12: Cleavage of FISH at varying concentrations of trypsin (increasing concentration from right to left including 1, 2, 4,8, 16 microliters each with time intervals of 0, 30, and 60min)

Using a 500mM NaCl, 25mM Tris at pH 8.5 buffer, the 5-LOX (12.1 mg/ml) OCTA dimer under went proteolysis with trypsin (.567 mg/ml). Trypsin cleavage inhibition was achieved by 300mM PMSF (phenylmethanesulfonylfluoride) in DMSO (dimethyl sulfoxide) at 0, 15, and 30minute time intervals. Additional similar experiments tested HCl (1M) and 10X-Beta-OG detergent as inhibitors for typsin. The protein to trypsin ratio was approximately 15.73: 1. This allowed effective cleavage of 5-LOX but not excessive. All samples were spun down in 10K micron membrane filters after inhibitor addition in order to concentrate sample.

Mass spectrometry analysis of 5-LOX proteoyzed samples concluded that the larger signals from the small protein cleavage fragments were interfering with efficient analysis of the signals from the C-terminal cleaved protein target. A nickel column procedure attempted to purify the C-terminal tail cleaved 5-LOX and flush all smaller fragments out of the sample.

Mass spectrometry analysis of the samples purified by the nickel column indicated that the analyzed enzymes: LOX dimer, monomer, and mutant S663D were clipped in two places.

79229.6 + 55.85 = 79395.45 -149 (N-terminal Met) = 79246.45 with Fe; 79190 without Fe

Fluorescence Analysis of 5-LOX with CPA

Using spectrofluorimeter analysis, amino acid concentration of protein sample after proteolytic cleavage was analyzed against known amino acid concentrations of isoleucine and alanine. The amino acid concentrations reveal the intensity at which 5-LOX was cleaved. The control samples of both isoleucne and alanine were made in decreasing concentration by serial dilution: 1mM, 500μM, 250 μM, 125μM, 62.5μM, 31μM, 16μM, 8μM,

4μM, 2μM, 1μM, 500nM, 250nM, and 125nM. TCA (Trichloroacetic acid) was found to lower the signal intensity of the amino groups.

The amino acid concentrations in solution were analyzed after exposing protein sample to carboxypetidase A (CPA) and testing with fluorescence the intensity and reproducibility of signal obtained. A buffer exchange from Tris-Cl to sodium phosphate was required due to Tris's interference as a primary amine in the function of the fluoraldehyde (OPA) fluorescent reagent. Each sample was analyzed in replicates of three. Running a spectrometric assay of CPA with Hippuryl-L-phenylalanine tested CPA's activity. Samples were spun in microcons and pipetted into plate wells for fluorescence analysis.

Amino Acid Fluorescence of 5-LOX and 15-LOX: Testing the amino acid cleavage concentration after cleavage of 5-LOX and 15-LOX by CPA.

Protein sample of each 5-LOX and 15-LOX were first run through the buffer exchange to sodium phosphate. Protein samples were concentrated in 10K microcons after proteolytic cleavage with time intervals (0, 15, 30, and 60 minutes) and inhibition by 1,10 phenanthroline monohydrate (60mM in ethanol). The signal intensities of each did not fully follow the control signals but were constant when the experiment was repeated. The fluorescence of different concentrations of CPA were analyzed and compared to enzyme cleavage results. It was concluded that the CPA was interfering with the signal of amino acid cleavage products of each protein.

Dynabead Pull Down and Western Blot Analysis of Pure OCTA and FISH Protein

Separate pure OCTA monomer protein samples were pulled down using "tail" and "body" antibodies. Elution samples from the pull down were run on a SDS gel. After transferring to membrane, the membrane was cut so that respective tail and body samples could be expressed in the appropriate antibody. The pull down samples were also run in

time intervals of 10, 30, 60 minute of protein Dynabead-antibody incubation. The tail antibody pull down decreased with time; however, the body antibody pull down over time was constant. Due to the appearance of smaller bands shown by the tail-expressed membrane, it was concluded that the tail target C-terminus was targeted. This can be further concluded by observance of the smaller bands seen in the "body" targeted protein.

The experiment was repeated only with the tail antibody for the pull down and expression. Protein bands came out lighter. The bands were still increasingly lighter with increasing protein incubation time intervals.

The maximal pull down was observed by running the pure protein on the SDS gel and examining it next to the tail antibody pull down samples. Also, non-heated and heated (completely denatured at 70˚C for 10 minute) tail pull down samples were compared. The heated samples were predicted to show higher intensity (darker) bands; however, the bands were lighter than the non-heated samples due to protein aggregation as seen in figure 13.

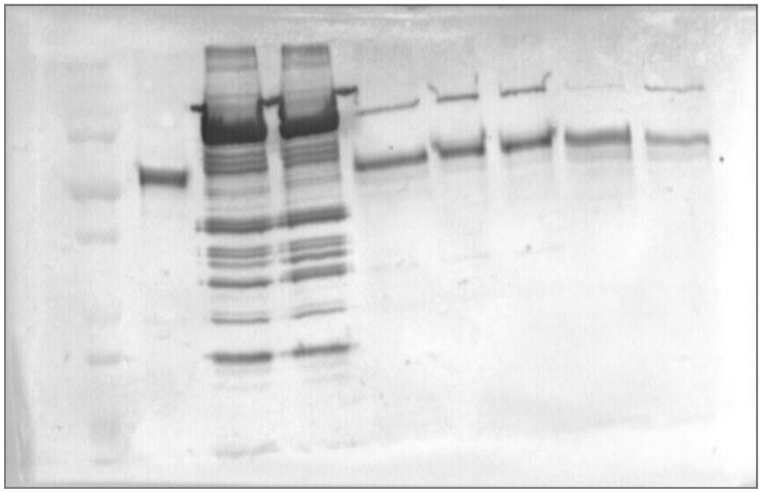

Figure13: Right most: Heated at 60, 30minute intervals; then tail antibody targeting and development at 60,30, and 0 minute intervals. Followed by tail targeted 60min supernatant, then pure OCTA protein followed by Tail antibody

Western Blot Analysis of Protein Lysate:

The OCTA lysate samples included heated and non-heated samples and were expressed in “tail” antibody. The lysate heated samples also demonstrated aggregation. The lysate also demonstrated little change across different time intervals.

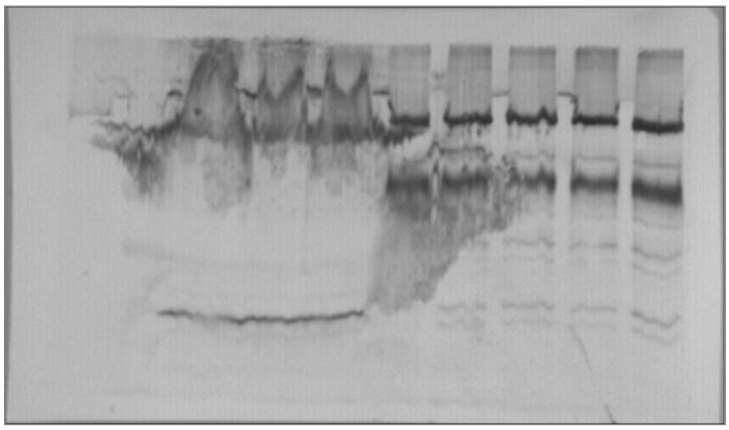

Figure14: First two wells from the right: AA (60min), AA (30min); next three: Tail antibody targeting and development at 60, 30, 0 minute intervals. Followed by AA 60min supernatant, Tail targeted 60min supernatant and pure FISH protein.

Due to the aggregation caused by heating the samples, AA was used to completely denature the protein and act as the control. AA proved to be a reliable control and the FISH samples demonstrated constancy with the control sample band intensities.

Western blot analysis next compared the OCTA and FISH proteins. Both proteins showed the same band intensity as the controls as can be seen in Figure 14. It was concluded that the protein was deactivating faster than it could be pulled down. Also, it was concluded that the tail antibody was targeting more than the tail through non-specific binding.

To test the reliability of the tail antibody, the experiment was run again where the protein was compared when expressed in the presence of tail peptide to protein not expressed in peptide as seen in figures 15 and 16.

The peptide proved that the antibody has a working target due to the lack of bands at the protein mark. In the membrane portion that was not expressed in peptide, the smaller bands seen may demonstrate non-specific binding.

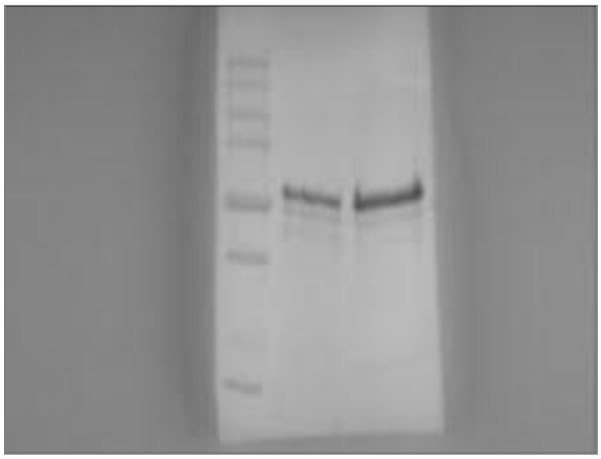

Figure15: left most: FISH then OCTA with tail antibody peptide development

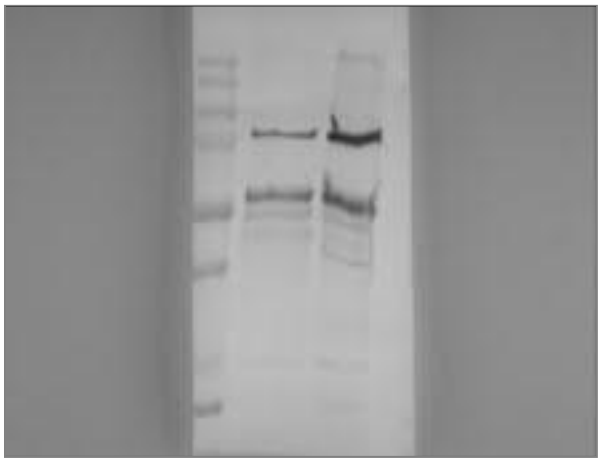

Figure16: left most: FISH then OCTA tail antibody development without peptide

VII. Discussion

The proteolytic cleavage of 5-LOX at its cut site in the C-terminus tail demonstrated the accessibility of the C-terminus tail on 5-LOX. The proteases processed the cleavage of several amino acid fragments, which were examined by molecular weight on an SDS gel. The mass spectrometry analysis examined the 5-LOX after proteolytic cleavage. Based on molecular weight mass spectrometry output for 5-LOX, the amino acids that were cleaved could be discerned. Knowing the C-terminus amino acid sequence, correspondence between the sequence and the amino acids cleaved could be examined.

The assumption made is that 5-LOX remains mostly in a folded inactive conformation; however, knowing that 5-LOX has a significantly fast turn over rate, 5-LOX's denatured conformation is assumed to be present along with both active and inactive states. The mass spectrometry results of the proteolytically cleaved 5-LOX allowed for analysis of what particular amino acids that were proteolytically cleaved. Nevertheless, these results did not confirm that the amino acids cleaved were part of the C-terminus tail but only that they were cleaved from 5-LOX.

The western blot technique would examine 5-LOX based on a C-terminus sequence specific antibody. Knowing that the antibody binds specifically to the C-terminus sequence when it is accessible, the pull-down and western blot procedure confirmed that the C-terminus was accessible. Additionally, the C-terminal tail should be more accessible to antibody binding in the soluble 5-LOX protein than in the stable 5-LOX protein. Yet again, due to 5-LOX's fast turn over rate the inactive and denatured forms cannot be distinguished by the "tail" antibody alone. The other "body" antibody recognizes and binds to an amino acid sequence on the surface of 5-LOX. This antibody would be able to recognize the active, inactive, and denatured forms. Nevertheless, this still leaves the inactive and denatured forms indistinguishable.

VIII. Conclusion

The next step in definitively indicating the presence of a C-terminus tail is intertwining the proteolytic assays with the western blot procedures. By running carboxypeptidase A proteolyticly cleaved 5-LOX on an SDS gel, the fragments cleaved will be separated. Then by developing the gel as a western blot with the "tail" antibody, it allows observation of just the C-terminus target sequence. Again, the soluble 5-LOX C-terminus should be more susceptible to proteolytic cleavage due to its greater instability. Therefore, fewer bands would develop in the western for the stable 5-LOX. This will more precisely define the difference in protease accessibility (the presence of an extended C-terminus tail) between soluble and stable 5-LOX. The cleaved 5-LOX developed with "body" antibody will act as a control giving a reference to the greatest intensity western band possible. Therefore, there would be band intensity differences between soluble 5-LOX and stable 5-LOX.

Another approach in determining the presence of an extended C-terminal tail is creating an “internal” antibody target sequence. Having an antibody that recognizes a sequence within the 5-LOX enzyme allows the active, inactive, and denatured conformations to be distinguished from each other. The “tail” antibody would recognize the denatured and inactive forms, the “body” antibody would recognize all conformations (denatured, active, and inactive), and the “internal” would recognize just the denatured conformation. Therefore, the “tail” targeted protein would have greater western band intensity than the “internal” targeted protein. The difference between these would demonstrate the relative intensity of a band representing just the inactive form. Additionally, the “internal” targeted band intensity will also differ between soluble and stable forms of 5-LOX.

These are the next steps in determining that the C-terminus does become unbound from the catalytic center and extend out to create an inactive conformation of 5-LOX. Through proteolysis and western blot development, differences in stability between stable and soluble 5-LOX can be confirmed as well as providing evidence of an inactive 5-LOX conformation.

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X. References

[1] Werz, Oliver, and Steinhilber, Bieter. "Therapeutic options for 5-lipoxygenase inhibitors." (2006): 701-718. *Science Direct.*

[2] Newcomer, Marcia E., and Nathaniel C. Gilbert. "Location, Location, Location: Compartmentalization of Early Events in Leukotriene Biosynthesis." *The Journal of Biological Chemistry* (2010): 1-6.

[3] Ford-Hutchinson, A. W., M. Gresser, and R. N. Young. "5-Lipoxygenase." (1994): 383-417.

[4] Gilbert, Nathaniel C., Sue G. Bartlett, Maria T. Waight, David B. Neau, William E. Boeglin, Alan R. Brash, and Marcia E. Newcomer. " The Structure of Human 5-Lipoxygenase." *Science* (2011): 217-219.

[5] Luo, Ming, Sandra M. Jones, Marc Peters-Golden, and Thomas G. Brock. "Nuclear localization of 5-lipoxygenase as a determinant of leukotriene B_4 synthetic capacity." *PNAS* (2003): 12165-12170.

[6] Dixon, R. A., R. E. Diehl, E. Opas, E. Rands, P. J. Vickers, J. F. Evans, J. W. Gillard, and D. K. Millar. "Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis." *Nature* (1990): 282-284.

[7] Rouzer, Carol A., Takashi Matsumoto, and Bengt Samuelsson. "Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A_4 synthase activities." Proc. *Natl. Acad. Sci USA* (1986): 857-861.

[8] Hanaka, Hiromi, Takao Shimizu, Takashi Izumi. "Intracellular localization of 5-lipoxygenase." *Elsevier Science B. V.* (2002): 343-347.

[9] Fischer, Lutz, Dagmar Szellas, Olof Radmark, Dieter Steinhilber, and Oliver Werz. " Phosphorylation-and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors." *FASEB* (2003): 949-951.

[10] McMillan, R. M. "Leukotrienes in respiratory disease." *Paediatric Respiratory Reviews* (2001): 238-244.

[11] Drazen, Jerffrey M., Chandri N. Yandava, Louise Dubé, Natalie Szczerback, Richard Hippensteel, Antonino Pillari, Elliot Israel, Nicholas Schork, Eric S. Silverman, David A. Katz, and Jeffery Drajesk. "Pharmacrogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment." *Nature Genetics* (1999): 168-170.

[12] Dahlén, Sven-Eril, Jakob Björk, Per Hedqvist, Karl E. Arfors, Sven Hammarström, Jan-Åke Lindgren, and Bengt Samuelsson. " Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: In vivo effects with relevance to the acute inflammatory response." *Proc. Natl. Acad. Sci USA* (1981): 3887-3891.

[13] Dahlén, Sven-Erik, Göran Hansson, Per Hedqvist, Thure Björck, Elisabeth Granström, and Barbro Dahlén. "Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with release of leukotrienes C_4, D_4, and E_4." *Proc. Natl. Sci USA* (1983): 1712-1716.

[14] Oldham, Michael L., Alan R. Brash, and Marcia E. Newcomer. "Insights from the X-ray Crystal Structure of Coral 8R-Lipoxygenase." *The Journal of Biological Chemistry* (2005): 39545-39552.

[15] Rådmark, Olof and Bengt Samuelsson. "5-Lipoxygenase: mechanisms of regulation." *The Journal of Lipid Research* (2008): S40-S45.

[16] Phillis, John W., Lloyd A. Horrocks, and Akhlaq A. Farooqui. "Cyclooxygenases,

lipoxygenases, and epoxygenases in CNS: Their role and involvement in neurological disorders." *Brain Research Reviews* (2006): 201-243.

[17] Rådmark, Olof, Oliver Werz, Dieter Steinhilber, and Bengt Samuelsson. "5-Lipoxygenase: regulation of expression and enzyme activity." *TRENDS in Biochemical Sciences* (2007): 332-341.

[18] Haeggstrom, Jesper Z. "Structure, Function, and Regulation of Leukotriene A4 Hydrolase." *American Journal of Respiratory and Critical Care Medicine* (2000): 171-177.

[19] "FDR-561-030U - Centrifugal concentrator Microcon YM-10 green 10,000 NMWL 0.5mL starting volume Millipore" https://extranet.fisher.co.uk/insight2_uk/getProduct.do;jsessionid=88348C0924EC6174FF7B57D629024B41.uklbhja34p?productCode=FDR-561-030U

[20] "NanoDrop 2000" http://www.nanodrop.com/Productnd2000overview.aspx.

[21] "Western Blot Equipment." www.bio-rad.com/blottingcells