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# Phosphorylation Studies of Serine 663 on Human 5-Lipoxygenase

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# Phosphorylation Studies of Serine 663 on Human 5-Lipoxygenase

by

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Undergraduate honors thesis under the direction of

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Abstract

This thesis describes the possible consequences of phosphorylation of serine 663 on human 5-lipoxygenase. Human 5-LOX is implicated in diseases such as asthma, heart disease, and rheumatoid arthritis. A phosphorylation mimic, a serine to an aspartate, was created. This phospho-mimic was found to change the specificity of the enzyme from a 5-LOX to a 15-LOX, and the two activities together result in synthesis of an anti-inflammatory product. To support the notion that aspartate is acting as a phospho-mimic, an actual phosphorylation was attempted. The phosphorylation was attempted *in vivo* with protein kinase A (PKA). PKA does not normally phosphorylate serine 663, but because it is a promiscuous enzyme, it was used in this study instead of the enzyme that phosphorylates serine 663 *in vivo*, extracellular signal regulated kinase 2 (ERK2). PKA was also readily available in a plasmid. PKA was expressed with a recombinant 5-LOX with other phosphorylation sites (serine 271, serine 522, and serine 523) mutated to alanine. I have not yet obtained evidence that S663 is phosphorylated.

I also began construction of a system for phosphorylation of 5-LOX by ERK2 *in* vitro. ERK2 is the enzyme that phosphorylates serine 663 *in vivo* and is activated by MAPK/ERK kinase 1 (MEK1). Purified MEK1 will be used to activate ERK2 and ERK2 will phosphorylate 5-LOX. Phosphorylated 5-LOX will be assayed to look for 15-LOX activity.

Finally, another phosphorylation mimic was made. Serine was mutated to glutamic acid, which could act as a better phospho-serine mimic because the glutamic acid side chain is closer to the length of a phospho-serine than aspartic acid. This construct has been made. The new mimic will be purified and assayed for 15-LOX activity.

## Introduction

5-lipoxygenase (5-LOX) is a member of the lipoxygenase family of enzymes that oxygenate polyunsaturated fatty acids to make a wide variety of lipid mediators. LOX enzymes from animals are non-heme iron containing enzymes that use arachidonic acid (AA), a membrane lipid, as a substrate; plant LOX enzymes use linoleic and linolenic acid as substrates. 5-LOX uses AA as a substrate to make lipid mediators involved in the inflammatory response. The specificity of the LOX enzymes depends upon which carbon is in closest proximity to the catalytic iron in the active site. To date five human LOX enzymes (5S, 12R, 12S and two 15S) have been identified. In addition, five other LOX enzymes (5R, 8R, 8S, 9S and 11R) have been identified in other species. Although LOX enzymes all catalyze the same oxygenation reaction, there is low sequence homology between the enzymes. Only the catalytic iron and three out of five amino acids that coordinate the iron are conserved (Schneider, Pratt et al 2007).

## 5-LOX Activation

Leukotriene biosynthesis begins when AA is cleaved from the cell membrane by cytosolic phospholipase $A_2$ ($cPLA_2$) when the concentration of cytosolic calcium increases from its normal low levels. Calcium is a potent cell-signaling mediator and is used in numerous cell-signaling events. Once arachidonic acid has been cleaved from the membrane by $cPLA_2$, it gets

sequestered by 5-lipoxygenase activating protein (FLAP) at the membrane. Without FLAP, there is little leukotriene production, so it is essential for optimal leukotriene production. 5-LOX, which can associate with the membrane when calcium levels increase, obtains AA from FLAP at the membrane and catalyzes formation of the 5S isoform of 5-hydroperoxeicosastetraeonic acid (5-HpETE). (Evans, Ferguson et al 2008; Plante, Picard et al 2006; Fig 3). 5-LOX and $cPLA_2$ both have calcium binding domains, called C2 or PLAT domains, that target them to the nuclear membrane to gain access to AA. Full activation of 5-LOX requires 4-10 micro-molar concentrations of calcium. About 2-3 calcium ions bind to 5-LOX in the C2 binding domain within the membrane associated beta-sheet domain. (Rogers, Inesi et al 1995; Kulkarni, Das et al 2002; Newcomer, Gilbert 2010).

## 5-LOX Mechanism

All lipoxygenase enzymes are non-heme iron containing enzymes. The iron plays an essential role in catalysis. The first step in activation of the enzyme is activating the resting iron from $Fe^{2+}$ (ferrous iron) to the oxidized $Fe^{3+}$ (ferric iron). The activation is done by lipid hyperoxides within the cell (Smith, Lands 1972; Schilstra, Veldink et al 1992). The resting catalytic iron is coordinated by conserved histidine residues and the C-terminal carboxylate. A water molecule coordinates the $6^{th}$ ligand position, creating an octahedrally coordinated iron (Fig

4). $Fe^{2+}$ reduces the lipid hyperoxide and becomes $Fe^{3+}$. A hydroxide ion is now coordinated to the 6th ligand of the iron (Minor, Steczko et al 1996).

Next, AA enters the active site ("head-first", with its carboxyl end entering first) placing carbon 7 (C7) in a specific place near the catalytic iron. The electron from the proS hydrogen on C7 goes to the d-orbital on $Fe^{3+}$ and through a proton couple electron transfer, gets placed on the hydroxide ion that is coordinating the 6th ligand, forming a water. This reduces $Fe^{3+}$ to $Fe^{2+}$ (Schneider, Pratt et al 2007; Vahedi-Faridi, Brault et al 2004). There is now a pentadienyl radical on AA that gets delocalized from C5 to C9. Molecular oxygen ($O_2$) enters into the active site on the opposite face from where the initial hydrogen was abstracted. $O_2$ then takes a hydrogen from the $Fe^{2+}$-$H_2O$, forming 5(S)-HpETE and reforming the $Fe^{3+}$-OH (Murphy, Gijon 2007; Fig 1; Fig 2). Then, using the same active site that makes 5(S)-HpETE, leukotriene $A_4$ ($LTA_4$) is made. The proR hydrogen from C10 is abstracted from 5-HpETE and also forms a radical, which localizes at C6. $Fe^{2+}$ then donates an electron and the oxygen – oxygen bond gets broken forming a triene epoxide and a free hydroxide anion. The free hydroxide ion then abstracts a hydrogen from the $Fe^{2+}$-$H_2O$ to restore the $Fe^{3+}$-OH. Lipoxygenase enzymes can make different products, depending on from which carbon the hydrogen gets abstracted (Schimizu, Izumiet et al 1986; Murphy, Gijon 2007; Schneider, Pratt et al 2007; Fig 2). After the 5-HpETE product is made, the product is used as a second substrate for 5-LOX to make leukotriene $A_4$ ($LTA_4$), which is the pro-inflammatory molecule (Gilbert, 2011).

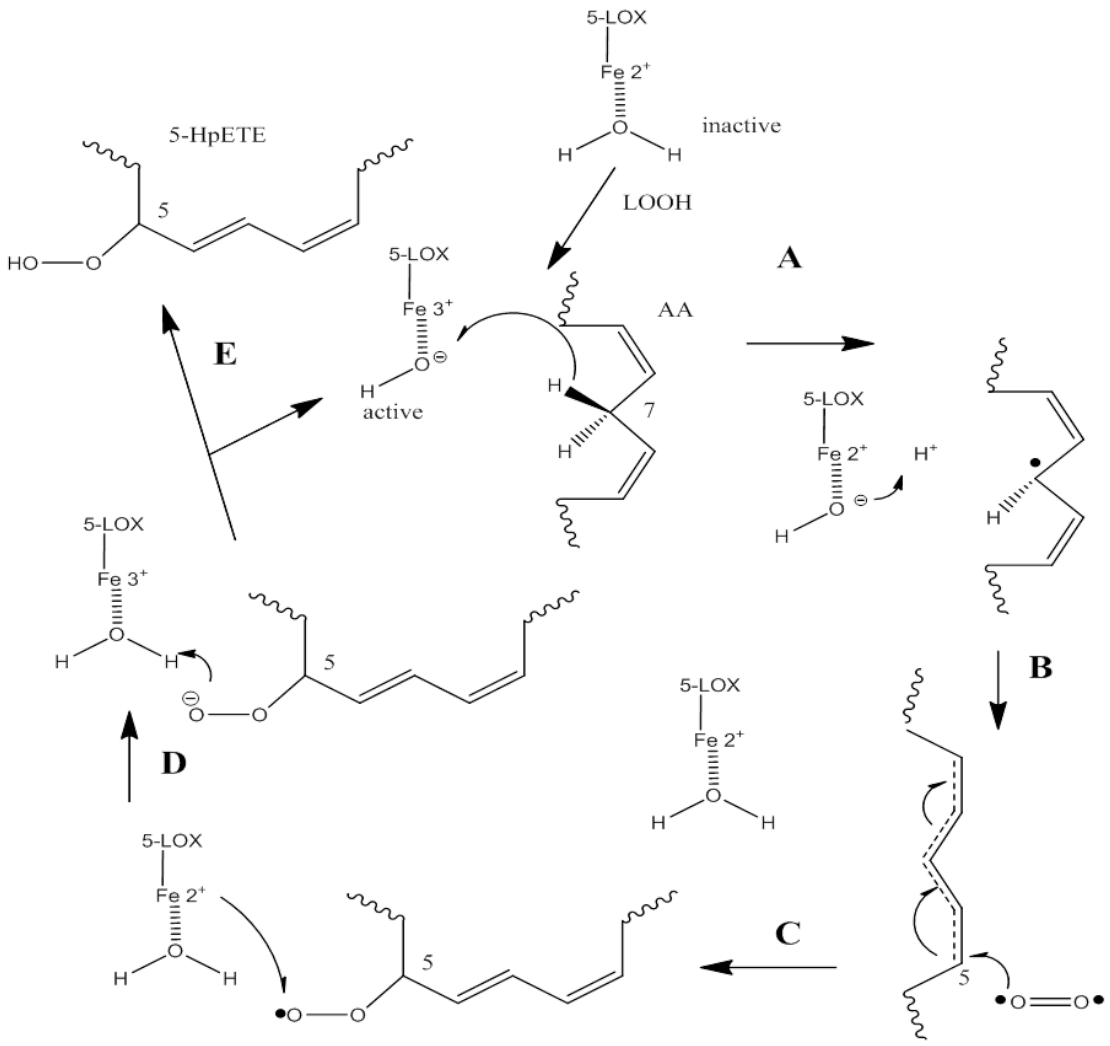


Figure 1. 5-LOX reaction mechanism. This shows the activation of 5-LOX, the catalytic iron, hydrogen abstraction, radical rearrangement, and formation of the 5-HpETE product. (Murphy, Gijon 2007)

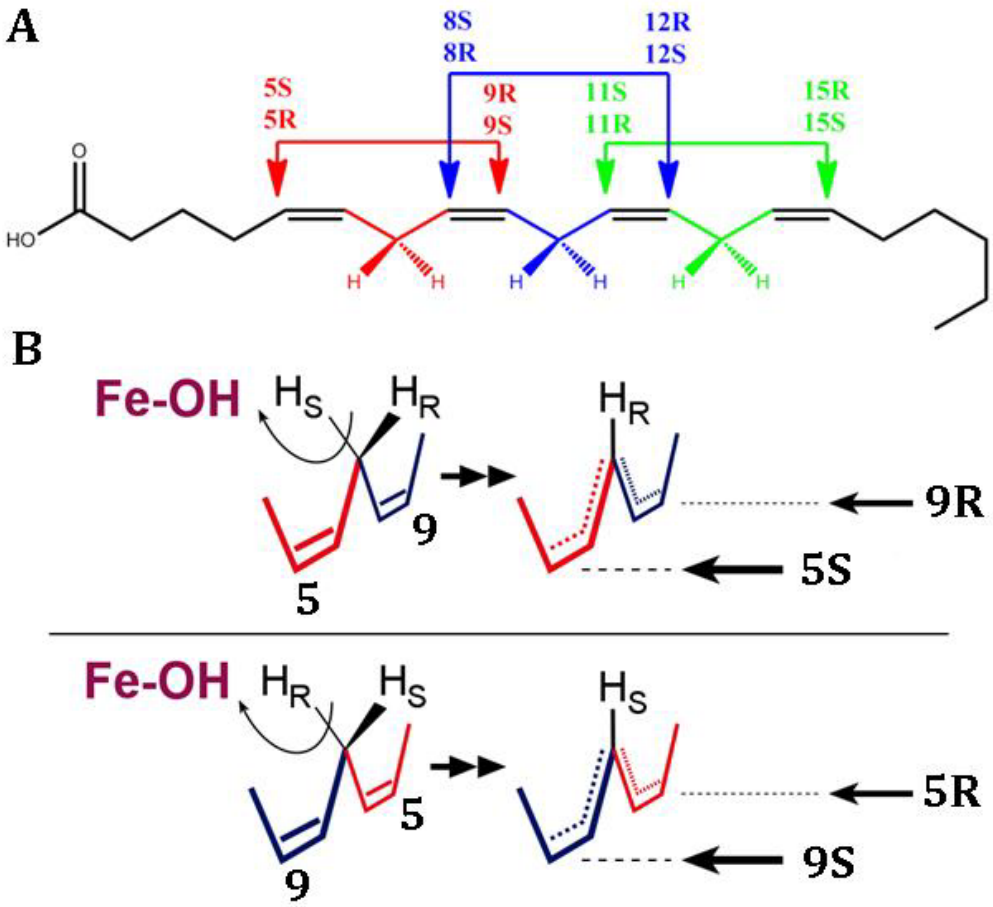


Figure 2. Lipoxygenase carbon specificity. There are six different hydrogens, shown in a color coordinated manner, available for abstraction from arachidonic acid to give a specific poduct,. For example, the hydrogen on carbon 10 can be abstracted to give the 8S, 8R, 12R or 12S product depending on from which side the hydrogen gets taken and from which side the oxygen is added. Taking the proS hydrogen from carbon 7 can only lead to either the 5S or the 9R product and taking the proR hydrogen can only give the 9S or the 5R product (Gilbert, 2011).

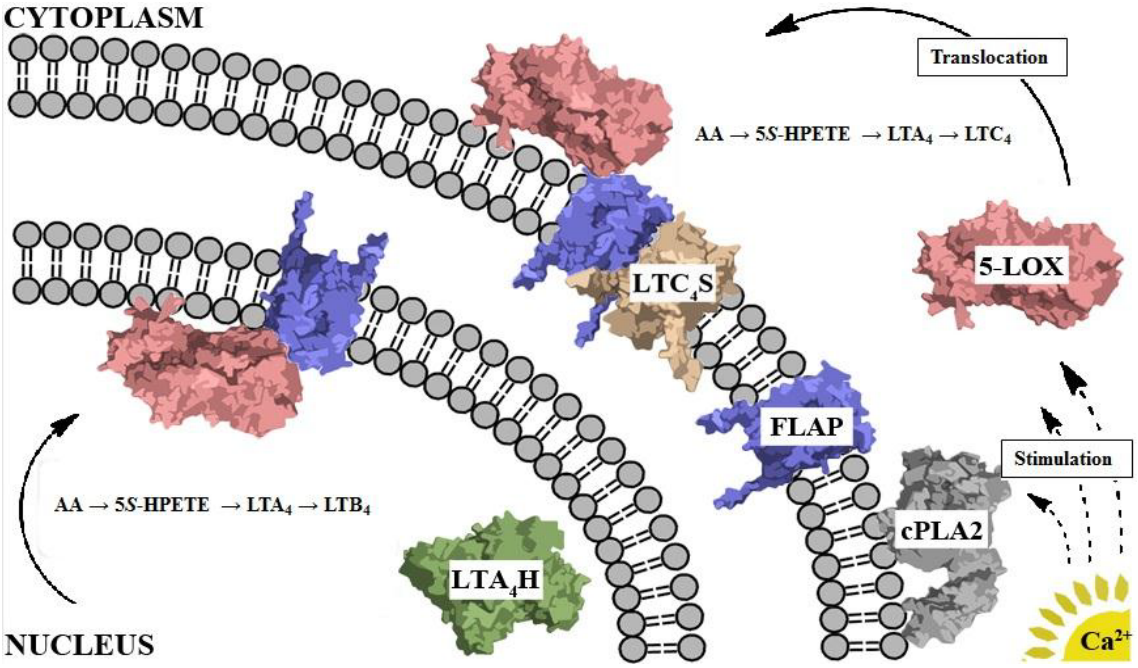


Figure 3. Leukotriene biosynthesis pathway. Calcium stimulation from a signaling even increases cytosolic calcium levels. 5-LOX is targeted to the membrane where it associates with FLAP and LTC4S. cPLA2 clips AA out of the membrane and gives it to FLAP (Gilbert, 2011).

## 5-LOX Inactivation and Phosphorylation

By the nature of the product 5-LOX produces, it is an inherently unstable enzyme. It would not be in the best interest of the cell to have excessive inflammatory products made, so the enzyme that makes these inflammatory molecules shuts off quickly after being activated. Overactive inflammatory responses have been linked to chronic diseases such as rheumatoid arthritis (Gheorghe, Korotkova et al 2009). One way to limit the amount of product made is to have an unstable enzyme that degrades quickly. The half-life of purified 5-LOX is about 24 hours at 2$^{o}$C. This could be caused by destruction or loss of the catalytic iron (Werz 2002; Percival 1991). The catalytic iron is susceptible to

reactive oxygen species, hydrogen peroxides and lipid hydroperoxides. All of these could lead to a loss of the iron (Percival, Denis et al 1992). In the presence of calcium, AA and phosphatidyl choline vesicles, 5-LOX has a half-life of about 30 seconds (Ueda, Kaneko et al 1986; DeCarolis, Denis et al 1996). This type of inactivation is called turnover-based suicide inactivation. Also, the products of the 5-LOX reaction can deactivate the enzyme (Aharony, Redkar-Brown et al 1987; Lepley, Fitzpatrick 1994). In addition, the iron could be irreversibly modified due to hydroperoxide intermediates (Radmark 2002).

One pathway for 5-LOX regulation is phosphorylation. There are three reported phosphorylation sites on the enzyme: serine 271, serine 523, and serine 663. Serine 271 (S271) is phosphorylated by mitogen-activated protein kinase activated protein kinase (MAPKAP); serine 523 (S523) is phosphorylated by protein kinase A (PKA); and serine 663 (S663) is phosphorylated by extracellular signal-regulated kinase 2 (ERK2) (Werz, Klemm et al 2000; Werz, Szellas et al 2002; Werz, Steinhilber 2006). Extracellular signals cause these phosphorylation events in addition to causing an increase in intracellular concentrations of calcium in a coordinated manner (Lepley, Muskardin et al 1996).

Unsaturated fatty acids stimulate the phosphorylation of S663 by ERK2. ERK2 can be stimulated by 5-α-dihydrotestosterone, which causes 5-LOX to localize to the nuclear membrane in non-calcium activated cells. This results in a decrease in leukotriene formation. Interestingly, asthma rates are higher in prepubescent boys and women of all ages because they have decreased levels of 5-αDHT (Pergola, Dodt et al 2008).

Phosphorylation at S523 by PKA leads to a decrease in 5-LOX activity. The phosphorylation site occurs at an allosteric regulation site. This site is near the 5-LOX nuclear localization sequence, which relocates it to the inner nuclear membrane (Lou, Jones et al 2004). Once phosphorylated, 5-LOX can no longer be sent to the membrane and therefore cannot be in close contact with FLAP to obtain its substrate. Addition of polyunsaturated fatty acids inhibits PKA phosphorylation, increases 5-LOX nuclear localization, and increases 5-LOX product formation (Flamand, Lefebvre et al 2006; Fig 4).

Serine 271 is right in the middle of a nuclear export signal. Phosphorylated 5-LOX on S271 holds the enzyme in the nucleus of cells. It is thought that S271 interferes with exportin-1-mediated nuclear export. Phosphorylation at this residue is a step in the regulation of leukotriene biosynthesis (Flamand et al. 2008).

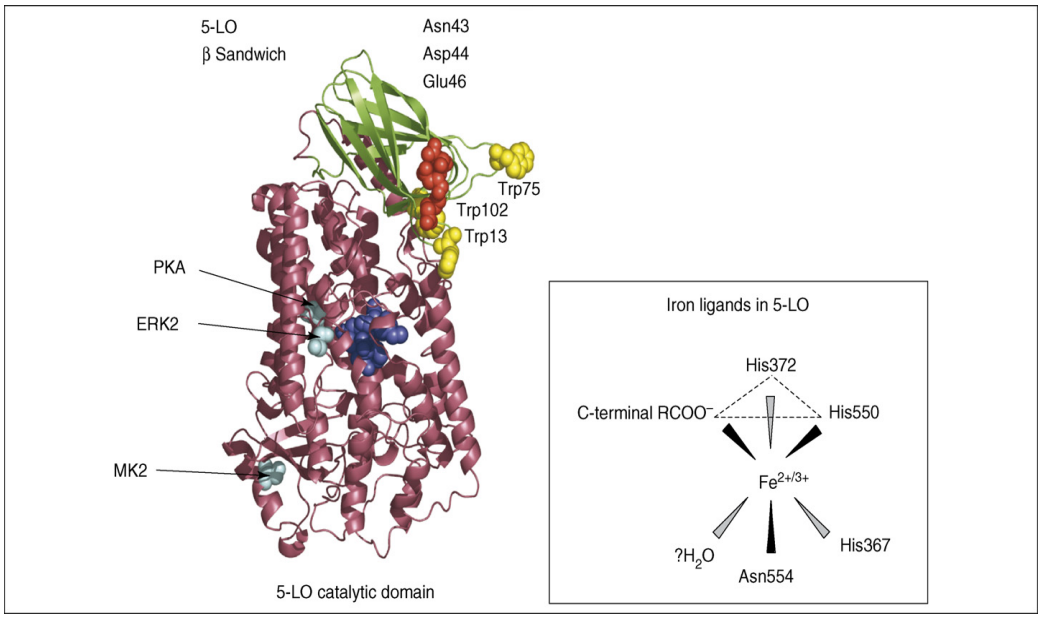


Figure 4. 5-LOX phosphorylation sites and iron coordination. On the left, the three different phosphorylation sites are shown. All three are within the alpha

helical catalytic domain. On the right, the residues that coordinate the catalytic iron are shown. His 372, 550 and 367, Asn 550, a water molecule, and the C-terminal carboxyl group coordinate the catalytic iron (Radmark et al, 2007).

## 5-LOXOCTA and 5-LOXOCTA-S663D

There are two domains on the 5-LOX enzyme – the C2-like calcium binding domain and the alpha helical catalytic domain (Chen, Zhang et al 1998; Chen Funk 2001; Hammarberg, Reddy et al 2002). The alpha helical domain contains the non-heme catalytic iron and is the site of catalysis. This iron is coordinated by histidines 367, 372 and 500 in addition to the main chain carboxylate end of the C-terminus (Minor, Steckzko et al 1996; Fig 4).

5-LOX is more unstable than the other LOX enzymes. Looking at sequences of other LOX enzymes reveals that 5-LOX has a region at the C-terminus that is lysine rich and adds to the instability of the enzyme. Leucine 655 normally is positioned in other LOX enzymes to bind to the catalytic iron, but in 5-LOX this residue is a lysine (Fig 5). There are also many salt links at the C-terminal helix that add to the stability of other LOX proteins, but these are not present in 5-LOX. Mutations were made in hopes of making 5-LOX more stable. Lysines 653-655 were replaced with the sequence from 8R-LOX: glutamic acid, asparagine and leucine (ENL). In addition, membrane insertion amino acids and a pair of cystines (Δ40 – 44GS, W13E, F14H, W75G, L76S, C240A and C561A) were mutated. 5-LOX with these mutations is known as 5-LOXOCTA, and is the mutant enzyme used for all studies in this thesis (Gilbert, 2011).

5-LOXOCTA has a 3°C increase in melting temperature and a longer half-life at 37°C, 16 hours as opposed to 7 hours, compared with the wild type enzyme. 5-LOXOCTA still makes the 5-LOX product, so these mutations do not interfere with the activity of the enzyme (Gilbert, 2011).

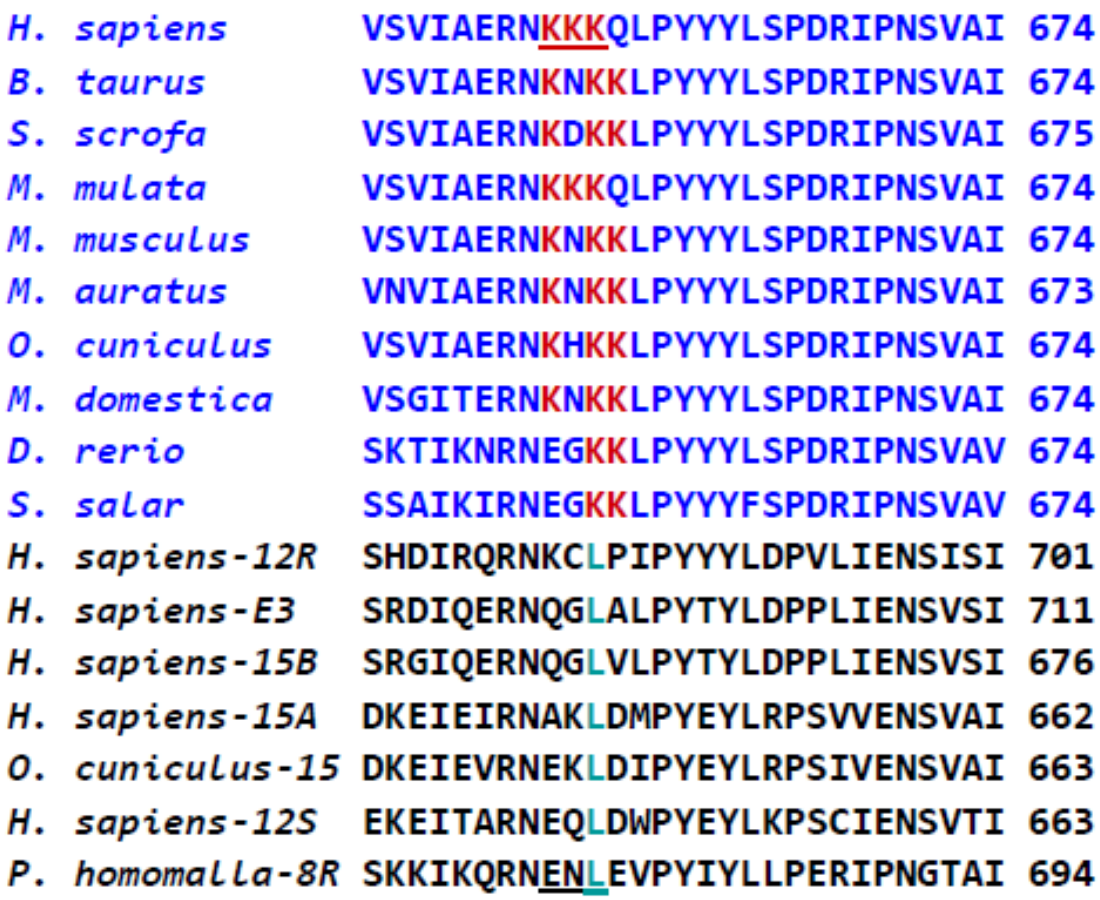


Figure 5. Lipoxygenase sequence alignments. 5-LOX sequences are compared to other LOX sequences. Where leucine exists (L) in the other LOX sequences, lysine (K) exists in 5-LOX. Sequence comparison lead to the 5-LOXOCTA, a more stable mutant (Gilbert, 2011).

Serine 663 is near the C-terminal end of the protein and is phosphorylated by ERK2 (Pergola, Dodt et al 2008). To study this site, S663 was mutated to an

aspartate, which has been seen as a sufficient mimic of phosphorylation (Gilbert, 2011; Huang, Erikson 1994). This mutation was found to convert the enzyme to a 15-LOX that is more active than 5-LOX. Leukotriene production is down regulated while lipoxin production is stimulated and the anti-inflammatory product is made (Fig 6). It has been thought that the production of the anti-inflammatory product is a trans-cellular reaction because its synthesis requires both 5-LOX and 15-LOX enzymes which are not present in the same cell types. The products of the 5-LOX reaction are substrates for the 15-LOX reaction, so the products from the 5-LOX producing cells would have to be transferred to the 15-LOX producing cells to make the anti-inflammatory product. This mutation suggests that maybe it is not a trans-cellular reaction, but that it takes place inside the same cell after a 5-LOX switching reaction (Gilbert, 2011).

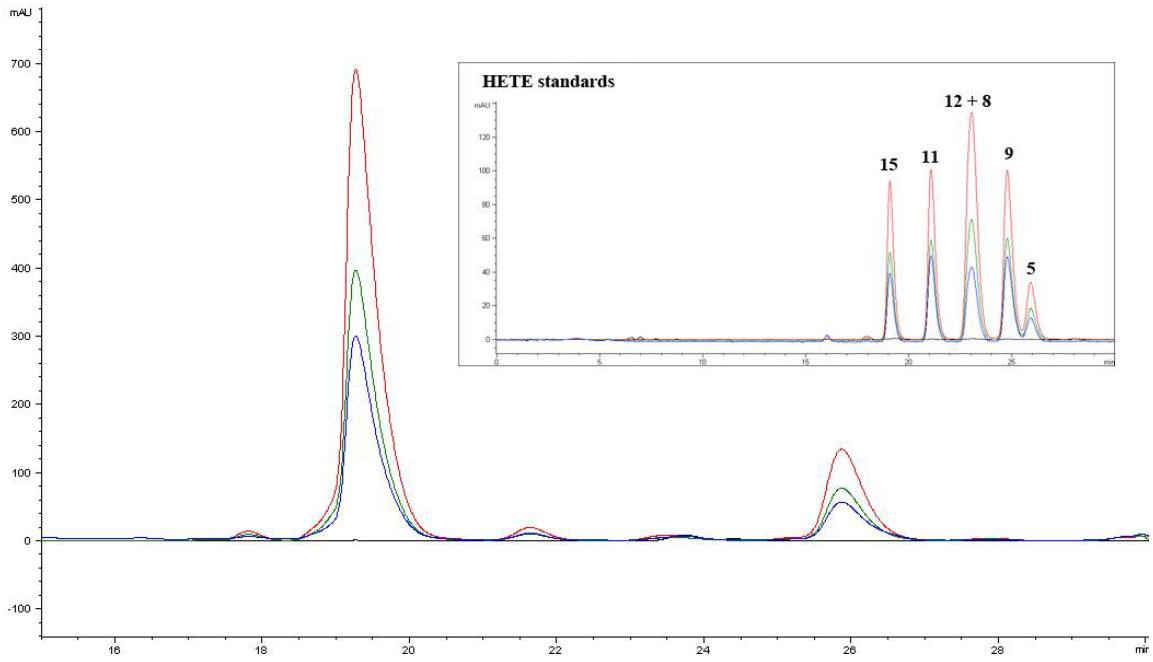

Figure 6. 5-LOXS663D assay. Elution time (seconds) vs. Absorbance units. This assay shows that 5-LOXOCTAS663D makes the 15S-HpETE product along with small amounts of 5-HpETE. (Gilbert, 2011)

In normal 5-LOXOCTA, AA enters the active site head first, placing C7 near the iron. 5-LOXOCTAS663D requires that the substrate enters the active site tail first and flipped. This must be the case because S663D makes the 15-SHpETE instead of the 15R-HpETE product. AA may enter the active site tail first due to charge repulsion between the aspartate and the oxygen on the head. This charge interaction may favor the tail first entry because there is no charge on the tail. 5-LOXOCTAS663D also makes the 15S-HpETE product much faster than the parent enzyme makes 5S-HpETE. This could be because the 15S-HpETE is the only product of the reaction and the enzyme doesn't catalyze another reaction like how 5-LOX makes $LTA_4$ after making 5-HpETE (Gilbert, 2011).

The structure of 5-LOXOCTAS663D with AA in the active site has been solved. There is a large conformational change in the structure of the enzyme that reveals an increased flexibility of the enzyme. Normally the alpha-2 helix and the arched helix are positioned to cover the active site, but in the S663D mutant, these two helices are displaced. This displacement gives a more exposed active site. In addition, residues 171 – 176 disappear from the electron density map, which means that they have lost defined structure and are now part of an undefined region. The first part of the alpha-2 helix is shorted and shifted and residues 190 – 194 and 204 – 215 are totally displaced. On the arched helix,

residues Leu414 to Gly428 can no longer be seen. All of these movements make for a very accessible catalytic iron (Gilbert, 2011).

Normally, S663 is at the end of a helix and can hydrogen bond as a hydrogen bond donor with Asp665. In the S663D mutant, the negative charge now causes charge repulsion with Asp665 and the residue moves closer to Asp666, which is in a Van der Waals interaction with Phe610. The alpha helix at the catalytic domain gets slightly shifted and Tyr605 bumps into the first segment of the active site-defining helix alpha 2, which causes it to shift. The arched helix then becomes disordered as well. There is a large conformational change due to a disruption of the hydrogen bond and Van der Waals network (Gilbert, 2011).

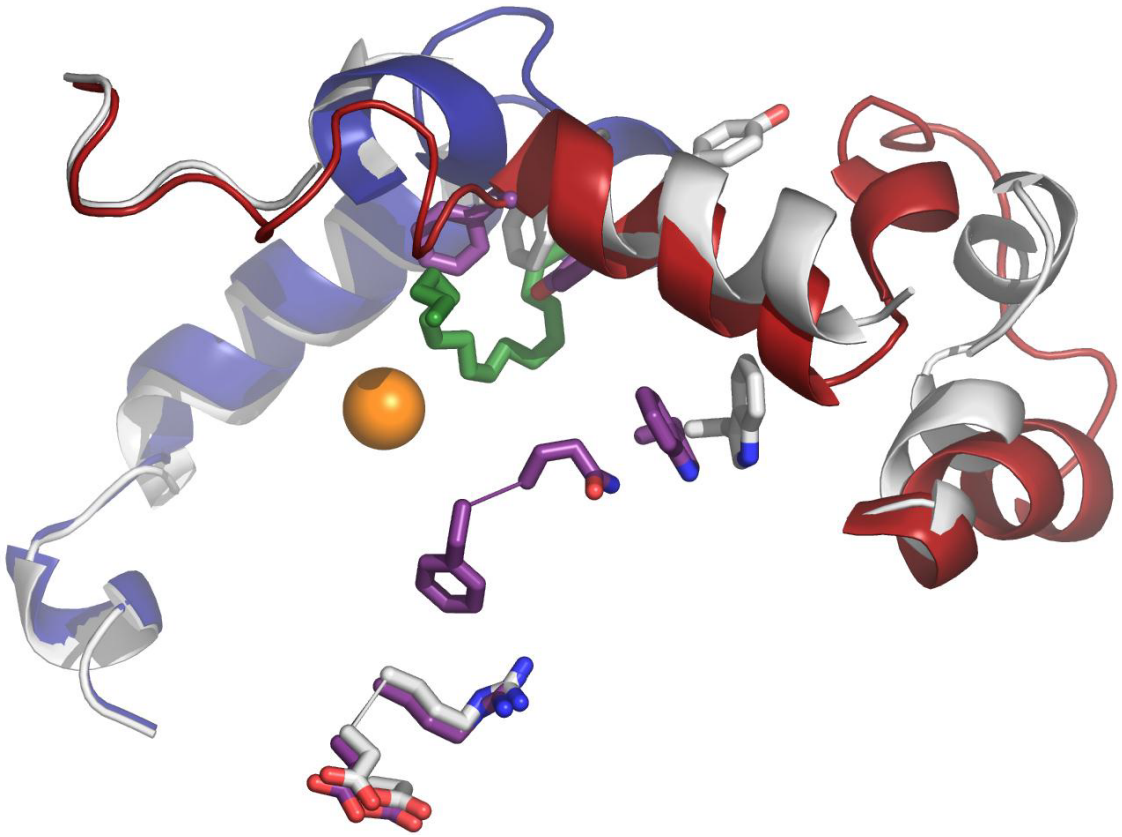

Figure 7. 5-LOXOCTAS663D structure comparison. Holo 5-LOXOCTAS663D (grey) with AA (green portion) and apo form (arched helix blue and alpha-2 helix red) superimposed. The grey portion protrudes out more in the arched helix and the alpha-2 helix (Gilbert, 2011).

## Thesis Project

This thesis focused on the phosphorylation of 5-lipoxygenase. In the first study, I co-expressed 5-LOXOCTA containing various mutations at potential phosphorylation sites with protein kinase A. The goal was to determine whether PKA could then phosphorylate S663 in *E. coli*. In the second study, I mutated S663 to glutamate rather than aspartate. Since glutamate has a longer side chain than aspartate, it could function more like a phosphorylated serine than aspartate. Finally, in the third study, I began efforts to express constitutively activated MEK1 and ERK2 in *E. coli* as C-terminal fusions to maltose-binding protein. This woud allow us to affinity purify the kinases, phosphorylate ERK2 *in vitro*, and then phosphorylated 5-LOXOCTA *in vitro*. Due to technical difficulties, none of these projects were completed, but my progress to date is described below.

The main goal of my project was to actually phosphorylate S663, determine its catalytic activity, and analyze the product of the reaction to see if it is the 5- or 15-LOX product. To do this, plasmid constructs were made with mutations at the other serine residues that are phosphorylation sites. S271, S522 and S523 were all mutated to alanines to prevent phosphorylation at those sites. S523 is the serine that is phosphorylated by PKA. Phosphorylation of

S271 in cultured cells is known to interfere with nuclear export, but activity of the enzyme phosphorylated at this residue has not been determined (Flamand et al. 2008). By mutating these residues to alanine, only S663, of the known targets for phosphorylation, could be phosphorylated.

The mutant protein (5-LOXOCTA/S271A/S522A/S523A) was inserted into a pET Duet1 that contained protein kinase A (PKA). PKA is a kinase enzyme that phosphorylates proteins that have certain consensus sites. The consensus motif for PKA is RRXS/TY where Y is a hydrophobic residue and X can be any amino acid (New England Biolabs). Even though PKA recognizes certain motifs, the sequence of the motif does not automatically make that site a PKA site. It does not take into account the three-dimensional structure of that site and the accessibility to the enzyme (New England Biolabs). Therefore, there may be different sites that PKA can recognize that are not consensus sequences. PKA phosphorylating non-consensus sequences has been seen before. For example, PKA phosphorylates acetylcholinesterase in vivo as well as 6-phosphofructro-kinase/fructose-2,6-bisphosphatase (PFKFBP) (Cavalier, 2012; Griffman, 1995). Cavalier et al. (2012) obtained 50% of PFKFBP phosporylated at a site not normally phosphorylated by PKA when the two enzymes were co-expressed in *E. coli*. Due to this, PKA was used as the enzyme to attempt a phosphorylation at the S663 site of 5-LOX OCTA in *E. coli*. ERK2 would be ideal to use because it is the kinase that phosphorylates that site in human cells. However, ERK2 has such low activity that it would take hours of incubation at 30 $^{o}$C to phosphorylate enough serine residues to be able to detect the 15-HpETE (or lipoxin) formation

by 5-LOX. This long incubation time could lead to 5-LOX inactivation. The purchase of sufficient amounts of ERK2 to phosphorylate 5-LOX rapidly is prohibitively expensive.

## Materials and Methods

### Construction of Mutant 5-Lipoxygenases

The plasmid used in all experiments was 5-LOXoctaS271A/S522A/S523A-PKA in pETDuet1. First, 5-LOXoctaS271A pET14b was made using whole plasmid PCR with 5-LOXocta as a template and PFU Ultra II HS polymerase (Stratagene). Next, 5-LOXoctaS271A/S522A/S523A pET14b was made using 5-LOXoctaS271A as a template in a whole plasmid PCR reaction. Finally, 5-LOXocta271A/S522A/S523A pET14b was transferred into PKA pET Duet using "cut and paste" method. The 5-LOX mutation was cut out of pET14b vector using Xba and Bgl II restriction endonuclease enzymes (New England Biolabs). PKA pET Duet was cut with Xba and BamHI (New England Biolabs) and the vector and insert were ligated together and used to transform α-Select cells (BioLine).

### Protein Expression and Purification

5-LOXoctaS271A/S522A/S523A – PKA pET Duet was transformed into Rosetta cells (Novagen) and expressed overnight on a small scale (3 mL) *via* autoinduction (Studier, 2005) before large-scale expression was attempted. This served to confirm that each mutant protein was expressed. Then 5-LOXOCTAS271A/S522A/S523A-PKA pET Duet was retransformed into Rosetta

cells (Novagen). Colonies were picked into 25 mL of LB and grown at 37 $^{o}$C at 220 RPM for 15 hours. Then 1mL of overnight was transferred to 100 mL of 2x YT media with 200 µL 1M $MgSO_4$. This was grown for 4 hours at 37 $^{o}$C and 220 RPM and then the temperature was turned down to 20 $^{o}$C for 29 hours. The cultures were then pelleted and stored at -80 $^{o}$C.

To purify 5-LOXOCTAS271A/S522A/S523A, cell pellets were resuspended in Bugbuster (Novagen, 2 mL/g) with DNase1 (2 Kunitz/g) and ~1.6M ProBlock inhibitor (Gold Bacteria 2D). After suspension, cells were lysed using a French pressure cell and then centrifuged at 40,000xg for thirty minutes. The supernatant was collected and then run on a 5mL HisTrap Global Cobalt column equilibrated in 50mM Tris pH 8.0, 500 mM NaCl and 20mM imidazole. Protein was eluted from the column with a linear gradient to 50mM Tris pH 8.0, 500mM NaCl and 200mM imidazole on an AKTA FPLC. Peak fractions were concentrated on an Amicon Ultra 30K (Millipore) to a volume of 1.5mL and then applied to a HiLoad 16/60 Superdex 200 column. Peak fractions were collected and equilibrated in 20mM Tris pH 8.0, 150mM NaCl, 5mM TCEP – HCl.

5-LOXOCTAS522A/S523A was also purified in a similar manner.

Assay for Activity

5-LOXOCTA mutants were assayed by monitoring the absorbance at 240nm for HpETE production and 270nm for leukotriene production in an Agilent 8453 Diode Array Spectrophotometer. 5-LOXOCTA mutants were assayed in a buffer of 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl and 10 mM AA (diluted from

a stock in 100% ethanol) with ~40 nM protein. Protein and buffer were blanked in the spectrophotometer and then AA was added to the cuvette and mixed. The absorbance was measured for 100 seconds.

## Results and Discussion

5-LOXOCTAS271A/S522A/S523A and 5-LOXOCTA/S522A/S523A were both co-expressed with PKA, purified, and assayed for activity (Figure 8). Only 5-LOXOCTAS522A/S523A had activity upon assay (Figure 9b). In this mutant, it is possible that serine 271 has been phosphorylated because it has not been mutated to an alanine. Because the basis of this experiment is the fact that PKA has been shown to be promiscuous, it is possible that serine 271 is also phosphorylated and affecting enzymatic activity.

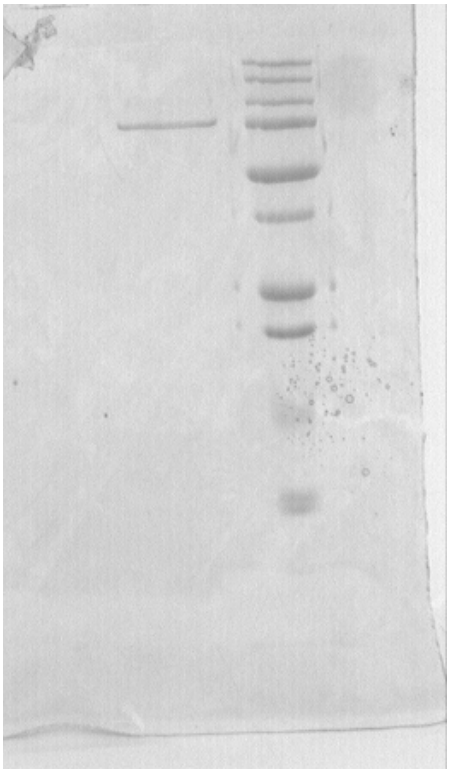

Figure 8. Purified 5-LOXOCTA/S271A/S522A/S523A. 5-LOXOCTA is about 75kDA.

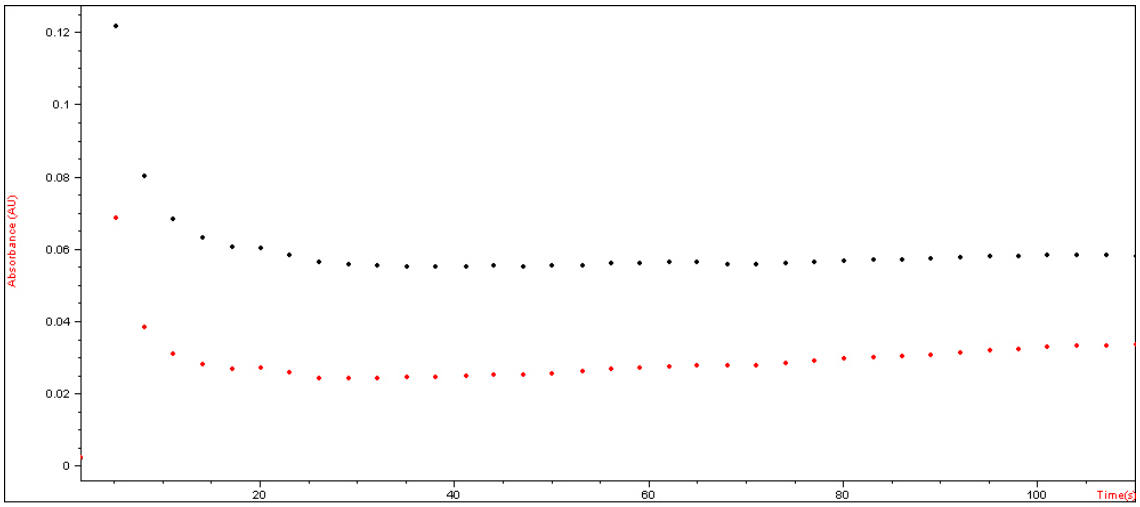


Figure 9a.

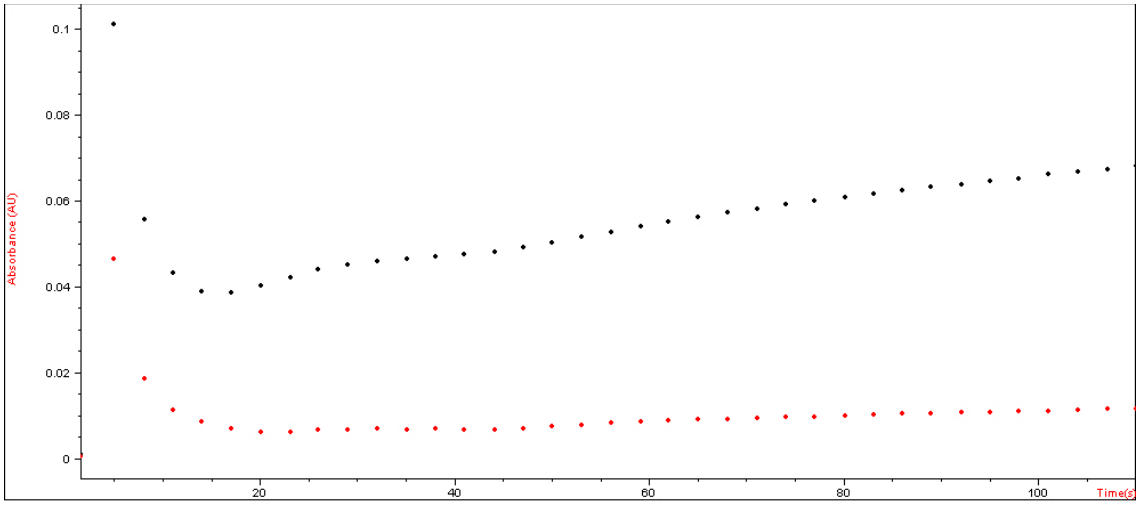


Figure 9b.

Figure 9a shows 5-LOXOCTAS271A/S522A/S523A assay. The black line represents formation of HpETE products (wavelength 240nm) and the red line represents formation of leukotrienes (wavelength 270nm). The beginning of the assay on the left hand side shows the initial mixing of the mixture in the cuvet. After the mixing, there is no increase in absorbance, showing that the enzyme was not active. Figure 9b shows 5-LOXOCTA/S522A/S523A assay. The initial peak at the left hand side represents the initial mixing of the cuvet. After mixing, the amount of HpETE product increased, as did leukotriene production slightly.

The product of the assay has not been able to be analyzed because the HPLC machine is currently broken. Even if the machine were working, if the 15-LOX product were detected, it would be inconclusive that this activity was due to serine 663 or serine 271 being phosphorylated. Thus, a new approach to phosphorylate serine 663 is being taken, but will not be able to be completed due to time constraints.

Because the 5-LOXOCTA/S271A/S522A/S523A mutant is inactive and the HPLC instrument is broken, I have started two new projects. The first project is an *in vitro* phosphorylation of 5-LOXOCTA by ERK2 and the second project is to make a better phosphorylation mimic of S663 by mutating the serine to a glutamic acid. None of these projects will be able to be finished due to time constraints, but the projects have been started.

## Phosphorylation of 5-LOXocta *in vitro*

### Background

This project will consist of phosphorylation of 5-LOXOCTA by ERK2, the protein that normally phosphorylates S663 in vivo. In the cell, ERK2 is an important protein involved in numerous signal transduction cascades. These signal transduction events are involved in cellular proliferation, differentiation and survival. Improper activation of ERK2 within cells is common in many types of cancer. The pathway which leads to ERK2 activation is a complicated pathway

that involves many upstream and downstream events. The pathway starts with an extra-cellular signaling event which activates a receptor tyrosine kinase and then eventually the protein Raf. Once Raf becomes phosphorylated, it activates the MAPK/ERK kinase 1 (MEK1) through phosphorylation. MEK1 must be phosphorylated at two serine residues, 217 and 221. Once activated, MEK1 can phosphorylate and activate ERK2. For full activation of ERK2, it must be phosphorylated at tyrosine 187 and threonine 185. ERK2 can go on and phosphorylate many different downstream target proteins, one of them being 5-LOX. Like any signaling pathway, the ERK2 pathway is regulated. Since ERK2 must be phosphorylated twice to be fully active, removing phosphates is a method of regulation. There are two separate phosphatase enzymes that dephosphorylate 5-LOX to turn down or turn off activity (R&D Systems).

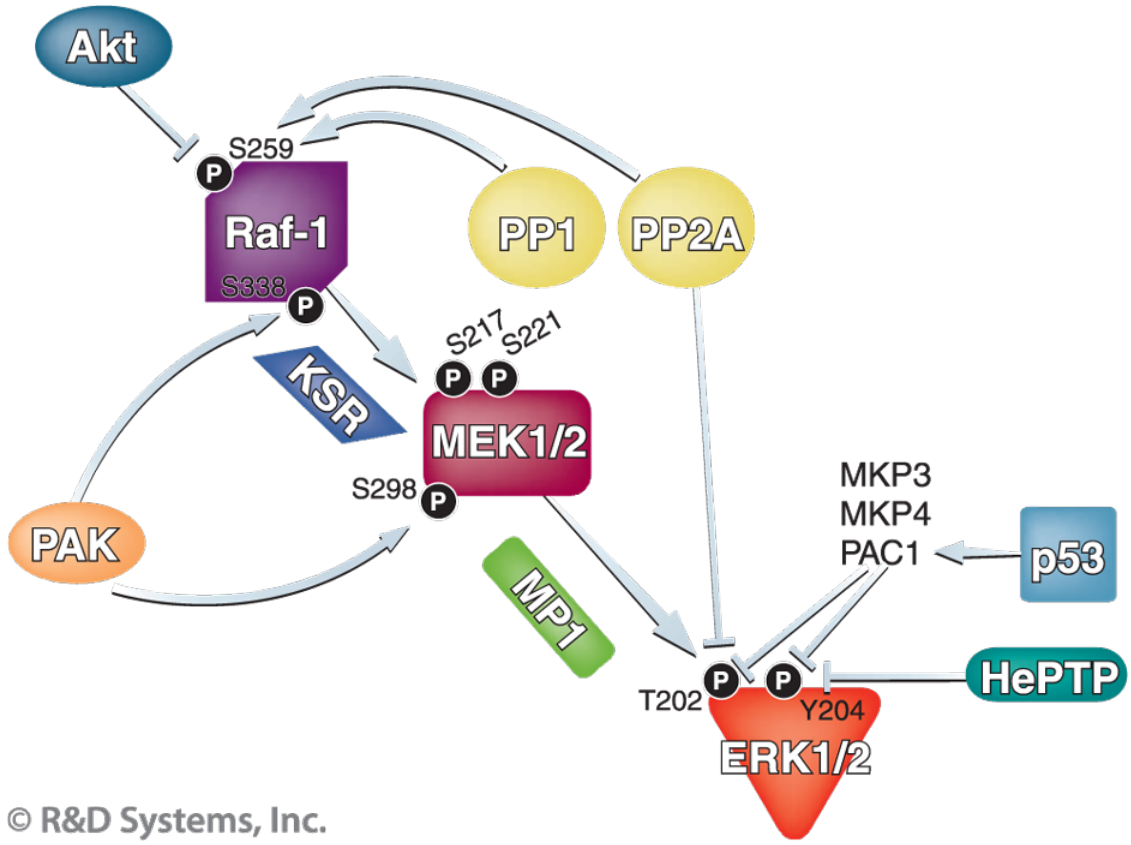


Figure 10. The ERK2 activation pathway. There are many steps involved in the final activation of ERK2 (R&D Systems, Inc 2004).

## Strategy and Rationale

The overall goal of this project will be to clone either MEK1 or ERK2 into pET Duet1-yjgD as a C-terminal fusion to maltose binding protein (MBP). The fusion proteins will be expressed and affinity purified and then incubated together in an appropriate buffer to activate ERK2. This mixture will then be incubated with 5-LOXOCTA to phosphorylate S663. Phosphorylated 5-LOXOCTA will be purified as described above for the mutant proteins and then an assay will be done for 15-LOX activity.

There are two reasons for constructing MEK1 and ERK2 as fusions to MBP. First, we can easily affinity purify them and separate them from his-tagged 5-LOXOCTA. Second, MBP has been shown to increase solubility of proteins expressed in *E. coli*. Presumably, it acts like a molecular chaperone by preventing aggregation of folding intermediates of recombinant proteins (Fox and Waugh, 2003).

The MBP fusions will be expressed in pET Duet1 yjgD. yjgD is a small, hyper-acidic protein from *E. coli*, which helps proteins fold and prevents protein aggregation within cells. It has been shown that increasing the amount of hyper-acidic proteins within the cell during expression of recombinant protein helps prevent protein misfolding and aggregation (Zou et al. 2011). By co-expressing human lipoxygenases with yjgD, personnel in the Newcomer and Bartlett groups have obtained excellent expression of all but one of them. By expressing ERK2 and MEK1 as MBP fusions and co-expressing them with yjgD, I hope to obtain a maximal yield of properly folded protein.

## Results and Discussion

I have started making the MEK1 and ERK2 constructs. Plasmids containing MEK1 or ERK2 cDNAs were purified from overnight cultures using a plasmid purification kit (Promega). Next, several attempts were made to amplify the inserts in the plasmids by PCR with PFU Ultra II HS polymerase (Stratagene) using primers designed to contain restriction sites for cloning and standard conditions. I did succeed in obtaining the MEK1 insert using an unorthodox

method (Figure 11). Prior to amplification with the standard PCR program, 5-cycles of PCR with an annealing temperature of 45$^{o}$C and an extension temperature of 62$^{o}$C were carried out. However, I still have not obtained the ERK2 insert.

The MBP insert has been amplified by PCR, digested with NcoI and BamHI, and is in the process of being ligated into pET Duet yjgD digested with the same enzymes using T4 DNA Ligase (New England Biolabs) The ligation will be transformed into α-Select cells (BioLine).

After creating these constructs, the next step will be to express and purify both ERK2 and MEK1 on an amylose resin. MBP will not only act as a chaperone for folding, but will also serve as a purification tool. MBP will bind to the resin and then will be eluted off with increasing concentrations of maltose.

Next MBP/MEK1 and MBP/ERK2 will be incubated together with Mg-ATP for about half an hour. MEK1 will not need to be activated because the construct being used has the two serine residues mutated to phosphorylation mimics, which makes the MEK1 constitutively activate. After ERK2 is phosphorylated, 5-LOXOCTA will be added and incubated at 30$^{o}$C, the optimal phosphorylation temperature, with about equal amounts of ERK2 to 5-LOXOCTA. As a control, 5-LOXOCTA would be incubated without ERK2 at 30$^{o}$C to make sure that the 5-LOXOCTA is not inactivated during the incubation. After incubation, the phosphorylated 5-LOXOCTA-ERK2-MEK1 mixture would be run over a nickel column to separate phosphorylated 5-LOXOCTA from ERK2 and

MEK1. This would need to be done because 5-LOXOCTA and ERK2 will be present in almost equal amounts and excessive amounts of ERK2 could interfere with the 5-LOXOCTA assay. After 5-LOXOCTA has been purified off the nickel column, it will be assayed with arachidonic acid to look for 15-LOX activity.

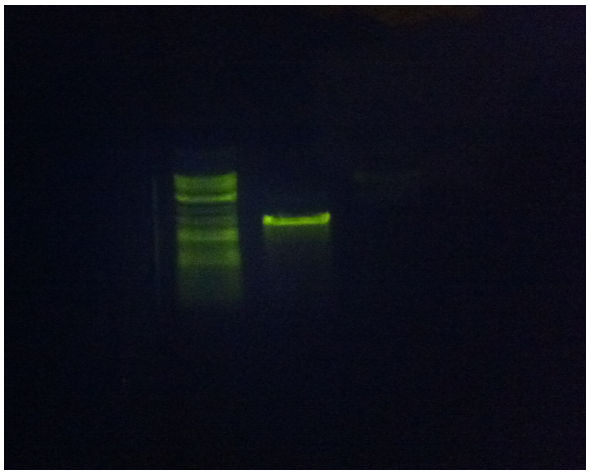

Figure 11. MEK1 PCR product.

Construction of another phosphorylation mimic of 5-LOXOCTA

Background

For the first phosphorylation mimic of 5-LOXOCTA, S663 was mutated to an aspartic acid. The negative charge of the aspartic acid mimics the negative charge of a phosphate group. Glutamic acid may act as a better phosphorylation mimic because it is longer and better imitates the actual phosphorserine.

Maciejewaki et al, 1995). This project will involve constructing S663E, measuring enzymatic activity, and analysis of the reaction product.

## Materials and Methods

5-LOXOCTAS663D was amplified by whole plasmid mutagenic PCR using PFU Ultra II HS polymerase (Stratagene) to make 5-LOXOCTAS663E. 5-LOXOCTAS663D was used as the template to give a diagnostic, which is the loss of a BamH1 restriction site. The PCR product was purified, ligated, and transformed into α-Select cells (Novagen). Plasmids were isolated from several colonies, digested with BamH1 and subjected to electrophoresis through agarose. Plasmids containing the S663E mutation will be used to transform Rosetta cells (Novagen) and the protein will be purified large-scale as described previously. The purified protein will then be assayed for 15-LOX activity.

## Results

The 5-LOXOCTAS663E mutation has been made. On the gel, S663D has an extra, small band and S663E is missing this band. The 2-log ladder did not run at the same level as the samples which we think is due to the fluorescent marker being too concentrated and causing the sample to not run properly. Since the ladder did not run at the same rate as the sample, there is no way to tell the size of these bands, but the small extra band should be 300 base pairs (Figure 12).

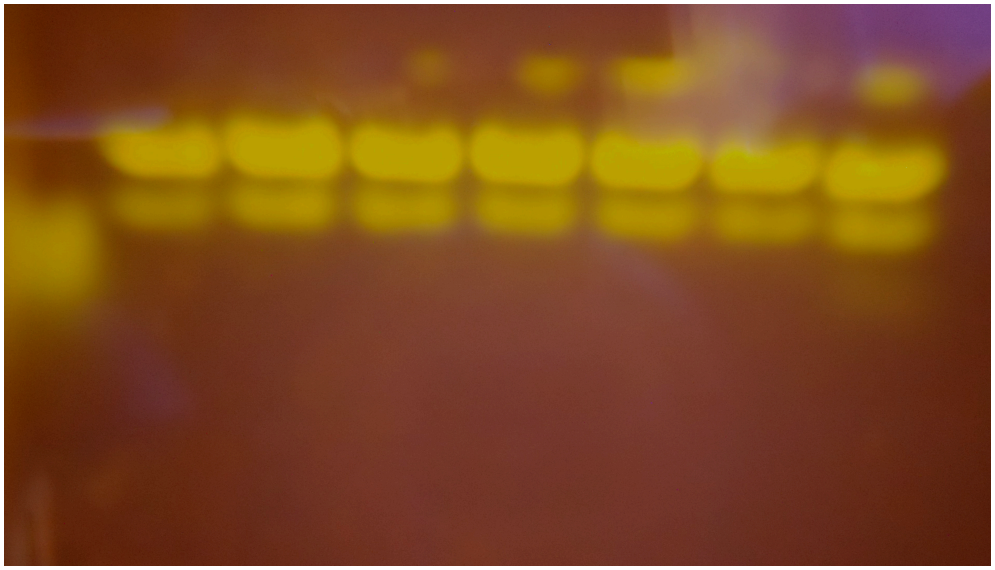

Figure 12. 5-LOXOCTAS663E. S663E is in lanes 2-6, S663D in lane 7 and 2-log ladder in lane 1. Lane 7 shows a very faint band under the first two bands. The S663E lanes do not contain this band.

5-LOXOCTAS663E will be transformed into Rosetta cells for expression. The enzyme will be purified and assayed as described in previous sections. In addition, the product will be analyzed by HPLC to determine whether it is 5- or 15-HETE.

## Summary

The phosphorylation of serine 663 was the focus of this thesis. After seeing 15-LOX activity from a 5-LOX phospho-mimic, an actual phosphorylation was attempted. Although ERK2 is the kinase that phosphorylates this residue *in vivo*, phosphorylation with PKA was attempted first due to the promiscuity of the enzyme. To examine just serine 663, other phosphorylation sites on 5-LOX were mutated to alanine (serine 271, serine 523, serine 522). Multiple constructs with combinations of these three serines mutated to alanines were made in pET Duet with PKA. Of these constructs, only 5-LOXOCTA/S522A/S523A was active. The

product of this assay could not be characterized because the machine to do the experiment was broken and was not fixed in time for me to complete the experiment.

Two other projects were started that would further characterize serine 663. The first was an *in vitro* phosphorylation of serine 663 by ERK2. ERK2 and MEK1 cDNAs were both purified and are in the process of being expressed and purified. Once purified, MEK1 and ERK2 will be incubated to activate ERK2 and then ERK2 will activate 5-LOXOCTA and the products of an assay with AA will be characterized.

A second phosphorylation mimic was made, mutating serine 663 to a glutamic acid instead of an aspartic acid. The mutant was made through mutagenic PCR and will be expressed, purified, and assayed for 15-LOX activity.

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