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INSIGHT INTO 5-LIPOXYGENASE BIOCATALYSIS AND INTERACTION WITH MEMBRANE COMPONENTS

Ramakrishnan B. Kumar

Karolinska Institutet

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Insight into 5-Lipoxygenase biocatalysis and interaction with membrane components

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ramakrishnan B.Kumar

Principal Supervisor:
Caroline Jegerschöld
Karolinska Institutet
Department of Biosciences and Nutrition
Division of Electron microscopy

Co-supervisor(s):
Hans Hebert
Kungliga Tekniska Högskolan
School of Technology and Health
Division of Structure Biotechnology

Agnes Rinaldo-Matthis
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Physiological chemistry II

Opponent:
Hartmut Kühn
Charité - Universitätsmedizin Berlin
Department of Charité Center Basic Sciences
Division of Biochemistry

Examination Board:
Pål Stenmark
Stockholm University
Department of Biochemistry and Biophysics

Lena Mäler
Stockholm University
Department of Biochemistry and Biophysics

Sven Hammarström
Linköping University
Department of Clinical and Experimental Medicine

“Work hard until you no longer have to introduce yourself”

Spector. H (Suits)

ABSTRACT

Inflammation is one of the innate defence mechanisms exerted by the human body for protection and to initiate the healing process. Chronic inflammatory reactions can lead to several disease conditions like asthma, allergic rhinitis and rheumatoid arthritis. A central role in these diseases is played by the Leukotriene (LT) family of the lipid mediators, one of family of pro-inflammatory lipid mediators derived from the 20-carbon fatty acid arachidonic acid. The key enzyme involved in LT biosynthesis is 5-Lipoxygenase (5LO) which initiates the leukotriene biosynthesis by catalysing the conversion of arachidonic acid (AA) to leukotriene A_4 (LTA_4). In this thesis, one aim is to elucidate the interactions of 5LO with the membrane and components therein which is the first step in the leukotriene biosynthesis and which could be one possible drug target. Furthermore, details of the LTA_4 formation were investigated as well as LTA_4 hydrolysis.

In **Paper I & II**, we demonstrated the calcium-mediated binding of 5LO with membranes *in vitro* using a membrane substitute called nanodisc. The selected nanodisc platform contained 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and the membrane scaffolding protein MSP1E3D1 which provides a bilayer surface of inner diameter around 10 nm. **Paper I** shows 5LO binds directly with nanodiscs in the presence of calcium. This calcium-mediated binding was shown by non-denaturing gel-electrophoresis. Due to the high binding affinity, the 5LO-nanodisc complex was visible in the gel in the absence of calcium ions during the gel run. In the presence of Ca^{2+}, a titration varying the ratio of 5LO to nanodiscs showed that maximum two 5LO binds to the nanodiscs used. The binding of 5LO with nanodiscs in the presence of calcium aides in the product formation and the results are comparable with the similar membrane mimic, liposomes. The complex of 5LO with nanodiscs was visualized for the first time by non-denaturing gel-electrophoresis and negative stain transmission electron microscopy (TEM). Furthermore, our results indicated that a 5LO dimer can be formed and it is formed by intermolecular disulphide bonds between cysteines located on the protein surface. The dimeric 5LO does not bind to a membrane and has no enzymatic activity. We have visualized also the dimer of 5LO by TEM. This paper clearly demonstrates the advantages of the application of nanodiscs as a membrane substitute to study monotopic membrane proteins. **Paper II** specifically focuses on a method developed to directly visualize and optimize conditions for the interaction of peripheral membrane proteins with the membrane using TEM. This method utilizes the phenomenon of stacking of phospholipids by a negative stain called sodium phosphotungstic acid (NaPT). Nanodiscs were used as a membrane substitute and form long stacks when stained with NaPT. The absence of stacking can be interpreted as a positive interaction between a protein and membrane, i.e. the protein bound to the nanodisc membrane prevents it from being stacked by the NaPT. With the help of this method, we have visualized different binding modes of 5LO with the membrane.

Paper III unravels the mechanism of product formation of 5LO by kinetic isotope effect. The results shed light on regiospecificity, H-transfer and donor-acceptor regulations in the oxygenation of AA.

Paper IV deciphers the secret behind dual product formation and reduced suicidal inactivation of *Xenopus laevis* (African clawed toad) leukotriene A_4 hydrolase, $xlLTA_4H$, by solving the structure by crystallography to a resolution of 2.3Å along with biochemical assays.

So far only one approved drug for clinical use is available that directly blocks the leukotriene biosynthesis, Zileuton™, whereas other drugs block receptors for leukotrienes. One reason for the lack of inhibitors may be the lack of detailed knowledge of the first steps in the leukotriene biosynthesis, something this thesis project initially set out to clarify.

LIST OF SCIENTIFIC PAPERS

I. Kumar RB, Zhu L, Idborg H, Rådmark O, Jakobsson P-J, Rinaldo-Matthis A, Hebert, H., Jegerschöld, C Structural and Functional Analysis of Calcium Ion Mediated Binding of 5-Lipoxygenase to Nanodiscs. PLoS ONE 11(3): e0152116. doi:10.1371/journal. pone.0152116 (2016)

II. B. Kumar, R., Zhu, L., Hebert, H., Jegerschöld, C. Method to Visualize and Analyze Membrane Interacting Proteins by Transmission Electron Microscopy. J. Vis. Exp. (121), e55148, doi:10.3791/55148 (2017).

III. Mittal M, Kumar RB, Balagunaseelan N, Hamberg M, Jegerschöld C, Rådmark O, Haeggström JZ, Rinaldo- Matthis A. Kinetic investigation of human 5-Lipoxygenase with arachidonic acid. Bioorg Med Chem Lett. 2016 Aug 1;26(15):3547-51. doi: 10.1016/j.bmcl.2016.06.025. Epub 2016 Jun 11.

IV. Stsiapanava A, Tholander F, Kumar RB, Qureshi AA, Niegowski D, Hasan M, Thunnissen M, Haeggström JZ, Rinaldo-Matthis A. Product formation controlled by substrate dynamics in leukotriene A4 hydrolase, Biochim Biophys Acta. 2014 Feb; 1844(2):439-46

CONTENTS

LIST OF ABBREVIATIONS

5HETE	5-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid
8HETE	8-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid
5(*S*)-HPETE	5-(*S*)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid
5LO	5 Lipoxygenase
13(*S*)-HPODE	13-(S)-hydroperoxy-9-cis-11-trans-octadecadienoic acid
AA	Arachidonic Acid
CLP	Coactosin-like protein
FLAP	Five lipoxygenase activating protein
KIE	Kinetic Isotope effects
LT	Leukotriene
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTA_4H	Leukotriene A_4 Hydrolase
LTC_4S	Leukotriene C_4 Synthase
MAPEG	Membrane Associated Proteins in Eicosanoid and Glutathione metabolism
MSP	Membrane scaffolding protein
NaPT	Sodium phosphotungstate
ND	Nanodiscs
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PDB	Protein data bank
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PTA	Phosphotungstic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
TCEP	Tris(2-carboxyethyl)phosphine
TEM	Transmission electron microscopy
xl	*Xenopus laevis*

1 INTRODUCTION & BACKGROUND

The human body works by several biochemical processes which control numerous pathways [1]. Various triggers can activate these pathways which leads to different biological responses [2]. Inflammation is one such response exerted by the human body upon interaction with harmful stimuli as a protective step to deter further interaction, damage and initiate the healing process [3]. In a nutshell, inflammation is an initial immune response and its function is to segregate, destroy and remove the damaged tissues (along with stimuli) by the interaction with harmful stimuli and restore the normal tissue function [4]. This immune response can be classified as acute and chronic inflammation [5].

Acute inflammation occurs within minutes on interaction with stimuli such as microbial infection. This infection triggers the releases of vasodilators by mast cells to increase the permeability of the blood vessels [6]. This aids the movement of immune cells like neutrophils to reach the interaction site through the capillary wall to neutralize the harmful stimuli [7]. Acute inflammation subsides on reducing/avoiding the interaction with the harmful stimuli.

The chronic inflammatory response is characterized by prolonged inflammation usually from weeks to months to lifelong. Chronic inflammation is transitioned from acute inflammation and its several characters are mentioned in Table 1. Acute inflammation initiates tissue repair process and in the case of chronic inflammation, this becomes chronically engaged leading to prominent structural distortion of the affected tissue [8]. This is mainly caused by persistent interaction with harmful stimuli or by auto-immune diseases, where the inflammation is triggered by the host [9]. During chronic inflammation, tissue repair and destruction takes place simultaneously.

Feature	Acute	Chronic
Onset	Rapid: in minutes or hours	Delayed: Days
Cellular infiltrate	Mainly neutrophils	Monocytes/macrophages and lymphocytes
Tissue injury, fibrosis	Usually mild and self-limited	Often severe and progressive
Local and systemic signs	Prominent	Less prominent to very prominent

Table 1: Features of acute and chronic inflammation [10]

There are several biochemical pathways involved in inflammation [3]. Leukotrienes (LTs) are one of the significant lipid mediators of leukocyte accumulation in acute inflammation [11] and also in chronic inflammatory diseases like asthma [12], allergic rhinitis [13] and rheumatoid arthritis [14].

1.1 LEUKOTRIENE BIOSYNTHESIS

Arachidonic acid (AA) is a polyunsaturated fatty acid of 20 carbons and is the precursor of LTs. It is an integral component of the biological membrane in the resting cell [15]. LT biosynthesis requires different proteins (Figure 1) to work in sequence, the first one being cytosolic phospholipase A_2 ($cPLA_2$) [16]. Upon stimulation, intracellular Ca^{2+} concentration increases which leads to movement of $cPLA_2$ from the cytosol to the nuclear membranes. There is also a slight structural change of $cPLA_2$ which increases its binding affinity towards phospholipids and hydrolyses the ester bond in the sn-2 position of phospholipids releasing the arachidonic acid [17].

Next coming into play in this concert is a protein called 5 lipoxygenase (5LO). The hypothesis is that the AA is now presented to 5LO by an integral membrane protein called 5-lipoxygenase activating protein (FLAP). The exact mechanism of and extent of involvement of FLAP are still not clear. AA is converted to 5(*S*)-hydroperoxyeicosatetraenoic acid (5(*S*)-HPETE) and then to Leukotriene A_4 (LTA_4) by 5LO. The LTA_4, the first leukotriene made, will be converted to Leukotriene B_4 (LTB_4) by Leukotriene A_4 hydrolase (LTA_4H) or Leukotriene C_4 (LTC_4) by Leukotriene C_4 synthase (LTC_4S) [18].

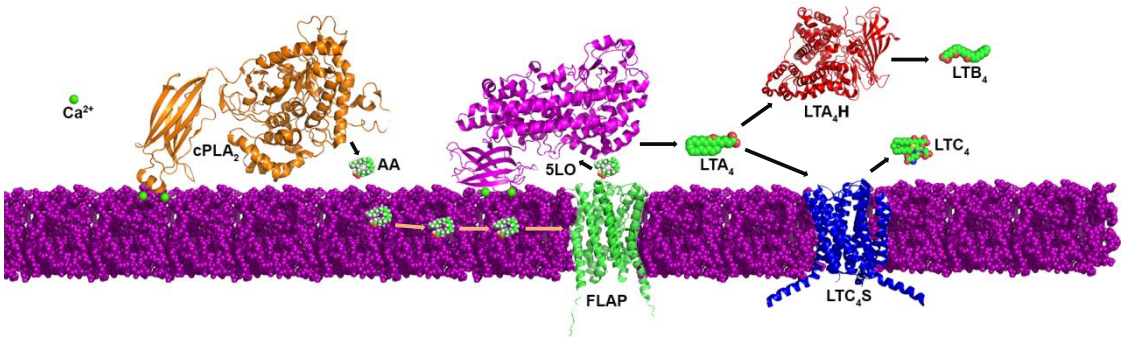

Figure 1: Initial stages in leukotriene biosynthesis

LTC_4 is converted to Leukotriene D_4 (LTD_4) by γ-glutamyl leukotrienase [19]. LTD_4 is converted to Leukotriene E_4 (LTE_4) by a membrane-bound dipeptidase by removing glycine [20]. These later leukotrienes C_4, D_4, and E_4 are termed "cysteinyl LTs" (cysLTs).

Higher levels of expression of 5LO and FLAP are seen in leukocytes [21]. A higher level of production of LTB_4 is seen specifically in neutrophils [22]. Cysteinyl LTs product levels are more pronounced in of eosinophils, mast cells, and basophils [23]. Macrophages and dendritic cells can synthesize all type of LTs. Non-leukocyte type cells like human bronchial fibroblasts can synthesize lower levels both LTs due to a lesser expression of 5LO/FLAP [24,25]. LTs can also be synthesized remotely in a non-leukocyte cell like an endothelial cell

by intercellular migration of LTA_4 synthesized from a leukocyte such as neutrophil, termed as transcellular biosynthesis [26]. Synthesized LTs are exported to the extracellular space by ABC transporters [27] and interact with specific G protein-coupled receptors that are situated on the outer plasma membrane of other cells [28].

	Receptors		
Cell Type	**BLT1**	**CysLT1**	**CysLT2**
Neutrophil	+	±	±
Macrophage or monocyte	+	+	+
Dendritic cell	+	+	?
Eosinophil	+	+	+
Basophil	+	+	+
Mast cell	+	+	+
B cell	?	+	?
$CD4^+$ T cell	+	+	?
$CD8^+$ T cell	+	?	?
Hematopoietic progenitor cell	?	+	?
Epithelial cell	?	+	+
Airway smooth muscle cell	+	+	?
Fibroblast	+	+	?
Fibrocyte	?	+	+
Endothelial cell	+	+	+

Receptor expression: positive (+), negative (−), minimal (±) or as yet undetermined (?)

Table 2 : Table for receptors in leukotriene biosynthesis [29]

Several receptors (Table 2) are involved in LT pathway and there are drugs (Table 3) available in market against different targets in leukotriene biosynthesis.

Target	Drugs
5LO	Zileuton [30]
BLT	U75302 [31], LY255283 [32] CP-105696[33]
Cys LT1	Montelukast [34], Zafirlukast[35], Pranlukast [36]
Cys LT2	BAY u9773[37,38]

Table 3: Anti-leukotriene drugs

1.2 COMPONENTS OF LEUKOTRIENE BIOSYNTHESIS

1.2.1 5-Lipoxygenase

5LO is a 78 kDa mobile protein (Figure 1 and Figure 2) and it catalyzes the conversion of AA to LTA_4. The structure of 5LO (PDB ID: 3O8Y) was published in 2011 [39]. It consist of a large C-terminal catalytic subunit composed of α-helices and contains a non-heme iron. A small N-terminal c2-like regulatory subunit constitutes a β sandwich. This regulatory domain of 5LO contains loops with the residues tryptophan 13, 75 and 102 involved in anchoring the 5LO to the membrane [40,41]. The rise in intracellular calcium concentration leads to binding of Ca^{2+} to the c2-like regulatory domain, which makes the enzyme to move from the cytosol to nuclear membrane. After binding to the nuclear membrane, 5LO binds with AA. This is followed by abstraction of pro-S hydrogen H_7 from C7 of AA and O_2 is introduced at C5 to form 5(S)-HPETE. The next is the formation of an unstable epoxide called LTA_4 by the abstraction of *pro-R* hydrogen H_{10} (from C10 position) causing dehydration of 5(*S*)-HPETE [42]. The active form of 5LO is a monomer and the existence of a dimeric form of 5LO was reported in 2011 [43]. Dimerization of 5LO was shown to occur by the formation of intermolecular disulfide bridges between cysteines C159, C300, C416 and C418 of the monomers leading to the dimerization. Bridges between these cysteines would lead to assembly in a head to tail orientation that would cover the catalytic iron binding site first shown 2011 [43] and later proved in cells 2015 [44]. This orientation of dimeric 5LO might hinder the substrate access portal leading to reduced production of LTA_4. The dimeric 5LO lacks enzymatic activity and cannot bind to a membrane *in vitro* [45].

There are several factors which can activate and aid the enzymatic activity of 5LO [46]. 5LO can bind to calcium and it is reversible. K_d for calcium is 6μM and two Ca^{2+} can bind to

specific tryptophans on 5LO in the membrane binding process [40,41,47]. Similar to 5LO, $cPLA_2$ contains a c2 like domain which binds to the membrane through tryptophans when these are bound to calcium. The release of AA from the membrane by $cPLA_2$ could be regulating the efficient utilization of 5LO [48]. *In vitro* studies indicate that membrane plays a major role in the regulation of the activity of 5LO. Cationic lipids are stimulatory and negatively charged lipids proved to inhibit the activity of 5LO [40]. The preferential binding of 5LO to the membrane is regulated by membrane fluidity rather than the composition of the lipids in the membrane. Membrane fluidity is increased by the presence of unsaturated bonds and in the case of 5LO, lipids with a high number of *cis* unsaturated bonds in the sn-2 acyl chain like the oleoyl in POPC or arachidonoyl in PAPC has shown to improve the activity of 5LO [46]. Coactosin-like protein (CLP) is a small 16 kDa F-actin binding protein that can bind to 5LO and the association increases the activity of 5LO in the absence of phosphatidylcholine (PC) and also it promotes the formation of LTA_4 in the presence of PC [49].

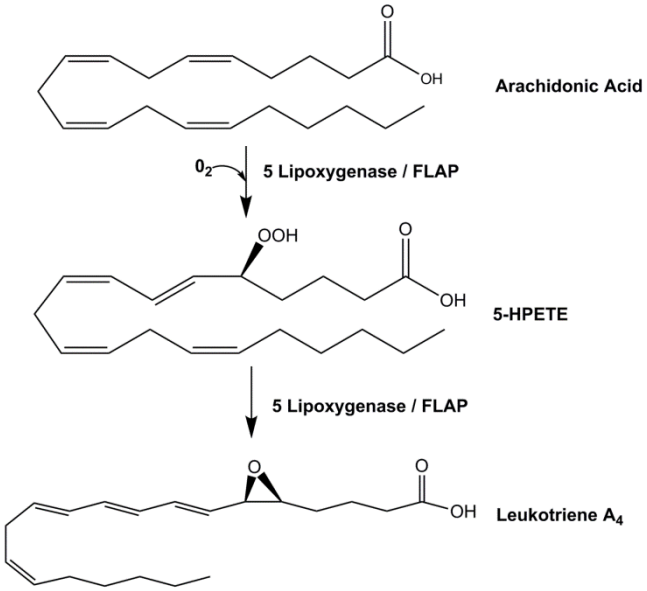

Figure 2: 5LO Mechanism for the formation of LTA_4 from AA via the intermediate 5(*S*)-HPETE. The proteins involved in each step are indicated.

Magnesium can stimulate the activity of 5LO *in vitro* with a K_d in mM range [50]. Even though ATP can bind to 5LO [51], it does not have any effect on its activity by itself. However, coupled with calcium, ATP has shown to improve the activity of 5LO [52]. In living cells, magnesium forms a complex with ATP as $MgATP^{2+}$ which is an activator of 5LO and could replace ATP [50]. The activity of 5LO is also regulated by phosphorylation at different sites [48]. Certain phosphorylation upregulates the activity of 5LO, other down regulate the activity and some affect localisation [53]. Phosphorylation at Serine 523 is regulated by protein kinase A (PKA). This phosphorylation has been reported to prevent nuclear localization and to reduce product formation in both *in vitro* and *in vivo* [54,55,56]. Phosphorylation on Serine 271 and 663 upregulates the enzymatic activity and the translocation of 5LO to the membrane. The former one is regulated by p38 kinase-dependent

MAPKAP kinases [57,58] and the later one by an extracellular signal-regulated kinase 2-dependent kinase (ERK2) [53,59].

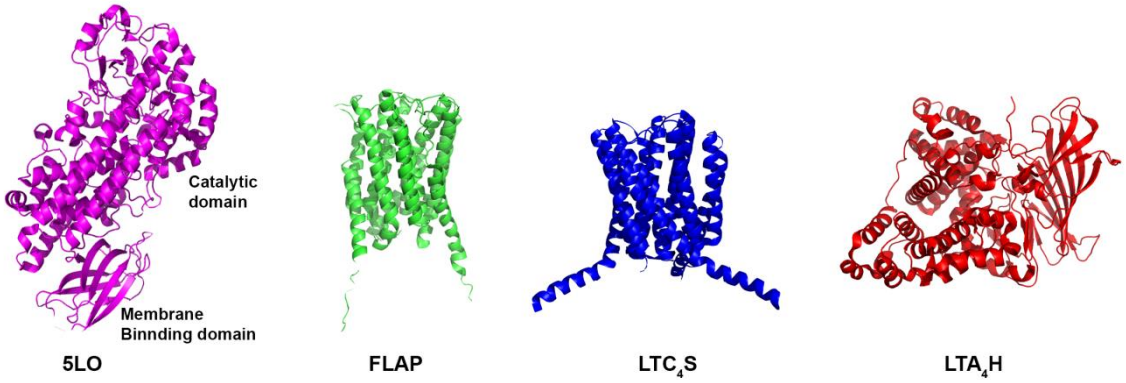

Figure 3: Enzymes involved in leukotriene biosynthesis

1.2.2 FLAP

Five Lipoxygenase Activating Protein (FLAP) is an integral membrane protein that belongs to the Membrane Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) superfamily [60,61]. It was identified as a 18 kDa protein bound to MK886 in a photoaffinity analysis [62]. The crystal structure of FLAP (PDB ID: 2Q7R) was solved and published in 2007 [63] to 4.2 Å (Figure 1 and 3). FLAP can bind to arachidonic acid [64]. The exact role of FLAP is still not clear. It is hypothesized to facilitate efficient transfer of AA to 5LO along with the conversion of 5(*S*)-HPETE to LTA_4 [65]. FLAP may also play a regulatory role by facilitating an initial supple complex formation with 5LO for efficient product formation and in a later stage, the complex becomes rigid leading to termination of activity of 5LO [66]. CLP forms a stable complex with 5LO which conserves the activity of 5LO [67]. This complex then moves from cytosol to the nuclear membranes where the complex anchors near FLAP which stabilizes the whole complex and facilitates the product formation [68].

1.2.3 Leukotriene C_4 Synthase

LTC_4S (Figure 1 and 3) is a homotrimeric integral membrane protein comprising 12 transmembrane α-helices [69]. It also belongs to MAPEG family [61]. The structure (PDB ID: 2UUH) was determined in 2007. It resides in the outer nuclear membrane and ER with the catalytic site facing the cytosol located in the interface between the monomers as is the case for all MAPEG trimers [70]. LTC_4S catalyses the conversion of LTA_4 to LTC_4 by the addition of glutathione [71]. This LTC_4 is exported extracellularly through multidrug resistance protein-1 (MRP-1) [72].

1.2.4 Leukotriene A_4 Hydrolase

As a bifunctional enzyme, human LTA_4H (Figure 1 and 3), has epoxide hydrolase activity which converts LTA_4 to LTB_4 , a potent inflammatory mediator and also an aminopeptidase activity [73]. The structure of human LTA_4H (PDB ID: 1HS6) was solved in 2001 [74]. As a

soluble enzyme with 69 kDa of molecular mass, the catalytic sites for both activities overlap and depend on a zinc atom [75]. The distinct feature of $hLTA_4H$ is the suicidal inactivation of both activities by covalent binding and modification of the substrate LTA_4 with tyrosine at 378 [76].

1.3 SPECIFICITY OF ENZYMES

Enzymes catalyze the conversion of substrates into products. The specificity of the enzyme makes the catalysis very efficient. Specificity of an enzyme can be categorized as in Chart 1.

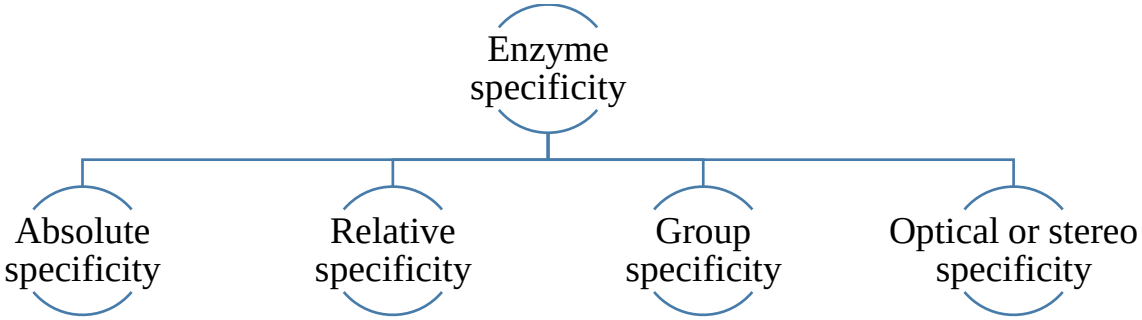

Chart 1: Different type of enzyme specificities [1]

Enzymes possessing absolute specificity can convert only one type of substrate (e. g. Lactase-Lactose). In the case of relative specificity, the enzyme can act on reactants belonging to a family of closely related substances (e.g. alcohol dehydrogenase - different alcohols). Group specificity is exhibited by an enzyme specific towards certain bond or certain chemical group (e.g. Trypsin-Amino group of lysine, arginine and histidine). Optical or stereo-specificity are characteristic of enzymes specific for a substrate with a specific optical configuration (e.g. α-amylase – α glycosidic bonds in starch).

An isomer is a molecule which possesses same chemical formula with a different chemical structure. In atomic terms, both molecules have the same atomic composition but differ in the spatial arrangement of atoms. Isomers can be classified as structural isomers and stereoisomers. Structural isomeric molecules are characterized by attachment of functional groups and atoms in different ways. In the case of stereoisomers the geometrical position of atoms and the functional group is different for the same molecule.

1.3.1 Lipoxygenases

Lipoxygenases are a class of enzymes which catalyze the peroxidation of polyunsaturated fatty acids. The products produced by the six human lipoxygenases are all stereo-regio specific [77]. The substrate AA has three sites for hydrogen abstraction and the products produced are either *R* or *S* isomeric state. Peroxidation of AA can be catalyzed by both 5 and 15 lipoxygenases and produces the corresponding *S*- isomeric peroxide [78] due to two different binding orientation of AA, either the carboxyl group enters first or the hydrocarbon enters first into the active site [79,80].

The 5LO is different from other lipoxygenases like 15LO, 12LO and 8*R*LO in several ways. First one is the ability of 5LO to convert 5(*S*)-HPETE into LTA_4 which is not seen in any other lipoxygenase both *in vivo* and *in vitro* [81]. This conversion of HPETE to LTA_4 is also seen in 15LO which can convert 15HPETE into 14, 15-leukotriene A_4, but it is only seen *in vitro* [82]. Other lipoxygenases do not require auxiliary proteins like FLAP [66,68] and CLP [68] to perform the catalysis efficiently as in the case for 5LO. The structure of 5LO published in 2011 [39] indicated the catalytic site of 5LO is completely enclosed which is distinct from other lipoxygenases [39]. The U-shaped active site is completely accessible in other lipoxygenase but in the case of 5LO (also Baltic coral 11*R*LO) the α-helix 2 is broken and becomes inaccessible due to the closure by aromatic amino acids [39,83].

1.3.2 Hydrolase

Whereas LTA_4H from human catalyses the conversion of LTA_4 to LTB_4, LTA_4H from *Xenopus laevis* ($xlLTA_4H$) can form two products (Figure 4) LTB_4 and Δ^6-trans-Δ^8-cis LTB_4 [84]. This new product is able to bind to the BLT1 receptor (see section 1.1 and Table 2) to a certain extent and can cause muscle contraction [85]. The crystal structure of $xlLTA_4H$ was solved in 2014 [84].

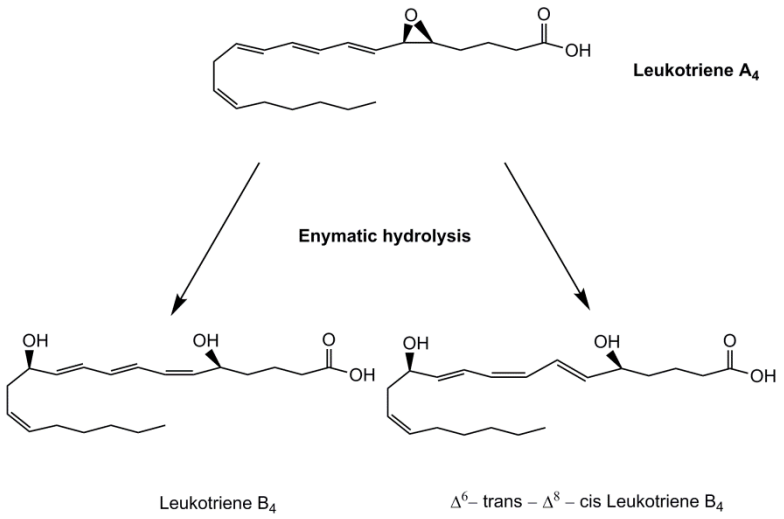

Figure 4: LTA_4H mechanism showing the two products formed, catalyzed by the leukotriene hydrolase from *Xenopus laevis*.

From the solved structure, it was evident that the catalytic site is too narrow to form both products. Triene C_4-C_{13} of LTA_4 is very flexible and it is altered during the catalysis. This leads to conformers of LTA_4. Conformers arise due to conformational isomerism where the isomers are formed due to rotations about the single bonds. From the crystal structure and conformer analysis *in silico*, it was suggested how the two products are formed by $xlLTA_4H$. The product formed depends on the conformational isomeric state of the substrate when it binds to the active site. Another reason could be phenylalanine 375 which reduces the substrate specificity of $xlLTA_4H$. The human LTA_4H cannot produce the second product, since the phenylalanine is replaced by tyrosine thus making the catalysis more specific.

1.4 CELLS

As the name signifies, leukotrienes are expressed in leukocytes [86]. These are white blood cells whose function is to defend the organism from infectious diseases and external agents [87]. Leukocytes are produced in hematopoietic stem cells in the bone marrow and these are present in all parts of the body [88]. They are broadly classified into five different types namely neutrophils, eosinophils, basophils, monocytes and lymphocytes. These leukocytes are mobile, seen in the blood and lymphatic system which is circulated throughout the body [89].

1.5 MEMBRANES & MEMBRANE MIMETICS

In above mentioned cell types and in any cell in general, membranes and membrane proteins play roles directly or indirectly in any given biochemical pathway to perform the function of the particular cell type [90]. They also play vital roles as structural support, in the selective passage of molecules, in signalling to initiate certain biochemical pathways etc [91]. In fact, leukotrienes are transferred across the cell membrane by transporters [72] to reach its corresponding receptor [92].

Membranes are bilayers made of two leaflets of lipids. The function of a given membrane is determined by the proteins embedded in the membrane and its lipids composition [93]. Phospholipids, glycolipids and sterols are examples of lipid classes with very different characteristics and structures (Fig. 5). The majority of bilayers contain a high concentration of phosphatidylcholine, a type of phospholipid carrying the zwitterionic headgroup choline [94] and more or less saturated acyl chains of varying lengths.

Sterols are mainly present in animal cells, e. g. cholesterol which confers membrane rigidity by promoting stiffer packing of phospholipid acyl chains. Glycolipids are normally located on the plasma membrane surface of the cell and functions as cell surface markers. They are made of various carbohydrate sugar chains.

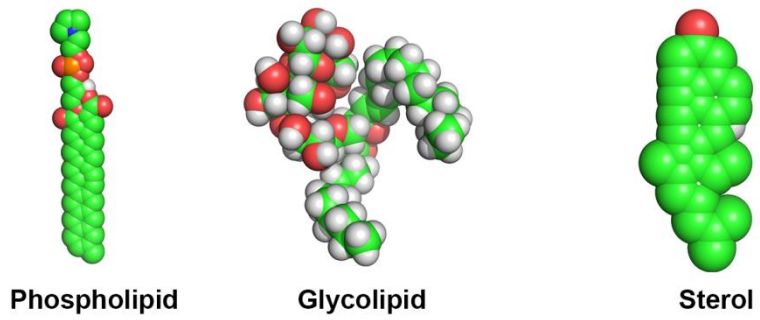

Figure 5: Different type of lipids

There are two ways to study the function of isolated membrane associated proteins (MAPs). The first one being isolation from the native membrane source directly. Such a methodology provides the most native form of the membrane and MAPs. Difficulties arise in identifying the native source that would provide amounts large enough to perform several experiments

and to purify the MAP to high concentration. An alternative method is to use membrane substitute systems to mimic membrane and furthermore that the proteins can be expressed by a recombinant expression system which would provide sufficient a quantity of protein. Relative ease in purification from recombinant sources is often found. However, the original membrane source of a MAP may require some extra consideration as to the choice of expression host and later in the choice of lipid mimetics. Selection of recombinant expression host depends on several biochemical characteristics of the protein of interest [95].

1.5.1 Detergent micelles

Detergents are amphipathic molecules similar to lipids but contain one hydrophobic tail and a hydrophilic head which makes their aggregation in water different from that of lipids described below (Section 1.5.2). When the concentration of detergent in a given system increases above certain concentration called critical micellar concentration [96], micelles are formed spontaneously. The micelles (Figure 6) are formed in a way that the hydrophilic region interacts with water and the hydrophobic tails, facing and covering the hydrophobic regions of the protein and forms a protein detergent complex. They are used for solubilisation/isolation of membranes and in purification of membrane proteins [97].

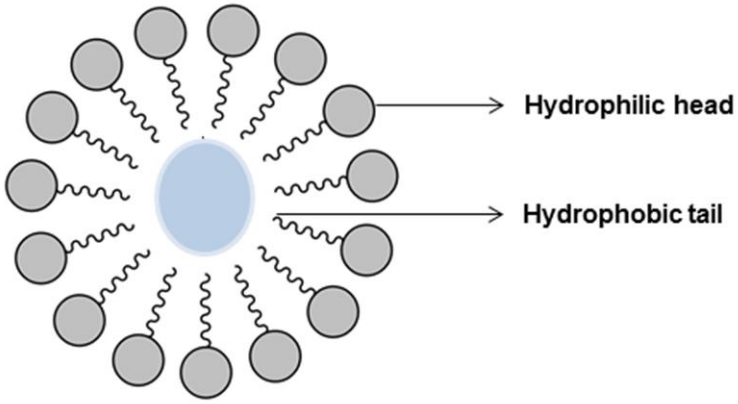

Figure 6: Detergent micelles

1.5.2 Liposomes

Liposomes are sphere shaped vesicles of lipids. These can be of a varied size from 2.5 nm to 2.5 µm. These can be produced by disrupting the native membrane which disrupts the structure even though the individual lipids maintain their character. This leads to reassembly into vesicles/liposomes denoted microsomes. Another method is to use the commercially manufactured lipids dissolved in chloroform, then dried to form a lipid cake. This cake is then hydrated with an aqueous solution and then agitated briefly to form liposomes [98]. Spherical objects like unilamellar small or giant vesicles or multilayered vesicles may form after sonication. Uniform vesicle sizes are obtained by extrusion of a solution of larger liposomes through a narrow pipe.

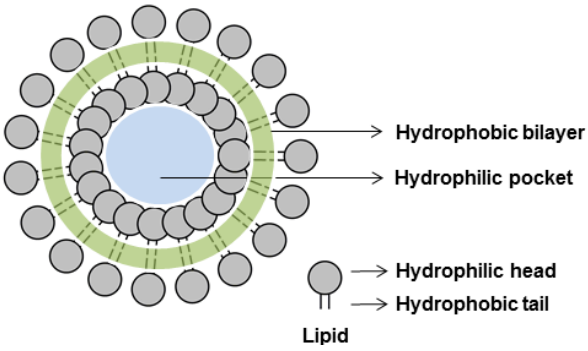

Figure 7: Liposome

The structure of liposome (shown in Fig. 7) makes it an interesting tool to use in biological sciences and in the medical field [99,100]. Its application is extensively researched in drug delivery due to the fact that it has a separate place for both hydrophobic and polar molecules in one structure [101].

1.5.3 Nanodiscs

Nanodiscs are assembled from two molecules of membrane scaffolding protein (MSP) enclosing phospholipids. The lipid molecules are non-covalently assembled like a bilayer and two molecules of MSP wrap around the hydrophobic lipid acyl chains in a belt like manner forming a nanodisc.

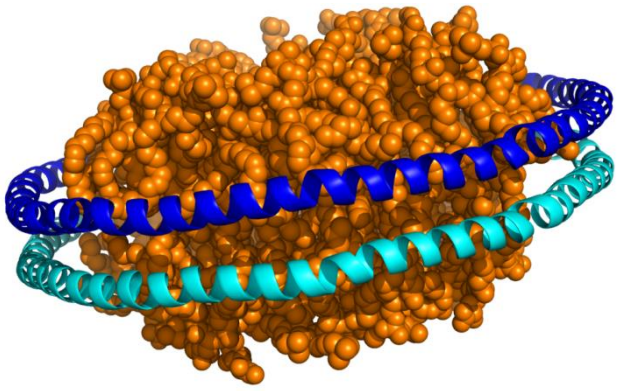

Figure 8: A nanodisc. Lipid core in orange. The two MSP proteins are coloured in light and dark blue. [102]

The MSPs are engineered versions of apolipoprotein-A1 and are made of 11-mer or 22-mer α-helical repeats punctuated with glycine or proline. The number of helical repeats determines the length of MSPs, hence the diameter of the nanodisc (10-20) [103]. As the MSPs have a soluble conformation in the absence of lipids, the nanodiscs were made by mixing MSPs and detergent-solubilized lipids in a defined ratio. This is followed by removing the detergent using bio-beads, upon which the MSP spontaneously rearranges with lipids to form nanodiscs [103]. The nanodiscs can be made as an empty nanodisc, i.e. containing only lipids encircled by the two MSPs, for use to mimic plain membrane. 5LO binding on a POPC bilayer is one example of this application (paper I and II). MSP and lipids can also self-assemble with a transmembrane protein, the most common use of the nanodisc

membrane mimic so far [102]. The nanodiscs are planar, both surfaces are accessible, are defined in size due to the choice of MSP length and can be quantified using the $UV_{280\ nm}$ absorbance of MSP which makes them a better membrane substitute in certain cases. Another more interesting feature is the option of selecting different MSPs (see Table 4) depending on the application and target. Is a membrane area around the transmembrane protein necessary for some reason? In this case, the nanodisc could be a good mimetic as the membrane area could have a predefined size that would allow precisely one interaction partner to bind both the transmembrane protein and embed on the membrane area. Such reconstituted membrane proteins in nanodiscs can be subjected to regular biophysical, enzymatic or structural experiments [104].

Here, the MSP denoted MSP1E3D1 was reconstituted with POPC to form a disc with an outer diameter of around 12 nm. This was used to investigate the membrane binding property of 5LO which is around 10 nm in length.

MSP	Disc diameter (nm)
MSP1D1dH5	8.2 (+/- 0.6)
MSP1D1	9.5 (+/- 1.1)
MSP1E3D1	12 (+/-1)
MSP2N2	16 (+/-1)

Table 4: Different MSPs and resulting nanodisc diameter [105]

2 AIM

The main aim of the thesis is to understand the mechanisms involved in leukotriene biosynthesis using several methods.

Papers I - III: Papers I - III: The crucial step in leukotriene biosynthesis is calcium-mediated binding of 5LO to the membrane. In paper I this interaction is characterised regarding its significant effects on enzymatic activity using biochemical assays and visualisation by TEM. Paper II aims to develop a TEM based method to visualise and analyse small membrane binding proteins using nanodiscs as membrane mimics. Paper III analyses the molecular steps involved in the catalysis of arachidonic acid by kinetic isotope effect (KIE) studies.

Paper IV: LTA_4H is the first enzyme which acts on the LTA_4 to form LTB_4. The human LTA_4H has a substrate induced inactivation which is not seen or reduced in lower organisms like *Xenopus laevis*. Furthermore, it forms LTB_4 along with Δ^6-trans-Δ^8-cis-LTB_4. This paper characterises the biochemical mechanism of catalysis of $xlLTA_4H$.

3 MATERIALS & METHODS

The methods used in this thesis which promoted understanding of the leukotriene biosynthesis are explained in detail in the "Materials and Methods" section of constituent papers. Briefly, some of the methods used in the paper I are described below.

3.1.1 Purification and stabilization of 5LO

Recombinantly expressed human 5LO loses its enzymatic activity within 10 hours after purification. To extend the enzymatic life span of 5LO, the following precautionary steps were taken. All the working buffers were extensively degassed and purged with nitrogen to remove oxygen. All the buffers were supplemented with 10 μ $MFeSO_4$ and 20 μg/ml catalase to prevent non-turnover based inactivation of 5LO [106]. For the experiments concerning monomeric 5LO, all the buffers were also supplemented with TCEP, a reducing agent to prevent dimerization of 5LO [107].

3.1.2 Calcium native PAGE analysis

The titration analysis performed in section 4.1.2 below, was done in sequential steps, the first one being an overnight equilibration of the native PAGE gel by a dummy run with running buffer containing 1 mM $CaCl_2$ and 5 mM β-mercaptoethanol at 30 V at 4°C. The separation run was performed the next day at 150 V, 4°C in the Ca^{2+} equilibrated gel.

4 RESULTS & DISCUSSIONS

4.1 PAPER I & II

5LO needs only Ca^{2+} to translocate to and bind to membranes. FLAP is not required to be present in the membrane for this translocation. The aim of the paper I was to characterise the membrane binding of 5LO in the absence of FLAP and to use nanodiscs as the membrane mimetic. The 5LO-ND complex formation was analysed by Coomassie blue stained non-denaturing polyacrylamide gel electrophoresis (blue native PAGE) and the enzymatic activity of 5LO was measured with UV-spectrometry and LC-MS/MS. The oligomeric state of 5LO was determined by blue native PAGE and its membrane associating capability and enzymatic activity was analysed by the above methods. All complexes were also analysed by negative stain TEM. Paper II is a continuation of Paper I. A method was developed to study membrane binding of peripheral membrane proteins using TEM and turning a particular negative stain induced artefact exerted on phospholipids into an advantage.

4.1.1 Initial binding analysis and oligomers of 5LO

The project started with the analysis of the interaction between 5LO and nanodiscs using blue native PAGE. The results showed that 5LO interacts with nanodiscs in the presence of calcium and a lack of binding is seen without calcium. The presence of 5LO was identified by excision of the band containing the complex of 5LO and nanodisc with calcium and running it on a denaturing PAGE followed by immunoblotting. An intriguing part of this experiment was the presence of a higher molecular weight band in the lane where only one band representing pure 5LO was expected. In 2011 Häfner et al. described the presence of dimerised 5LO due to inter-protein disulphide bond formation between 5LO´s cysteines, C159, C300, C416 and C418, located on the surface [43]. On examination of the 5LO SEC column eluate, showing two peaks, the former peak presented a retention volume corresponding to a weight twice the molecular weight of 5LO (78 kDa). The fractions were analysed in denaturing PAGE and immunoblot analysis which showed the presence of 5LO in both fractions indicating the former peak being a dimer of 5LO and the later one being the monomer of 5LO. The TEM analysis showed different orientations of dimeric 5LO. The dimer formation was prevented by using a reducing agent TCEP in all the working solution [45].

4.1.2 Analysis of monomeric 5LO

Initial analysis of the calcium-mediated binding of 5LO with nanodiscs using blue native PAGE showed a positive association. A titration analysis was performed in order to learn if the calculated size of the nanodiscs is sufficient for binding of 5LO and if both sides of the nanodisc would be used for binding. In the initial analysis, the observed band was weak due to the absence of Ca^{2+} in the gel which is required by 5LO for binding. Hence titration analysis was performed on a native PAGE equilibrated with Ca^{2+} before the separation run. The titration analysis of different ratios of 5LO and nanodiscs showed that a maximum of

two monomeric 5LO can bind to one disc. We could also see the 5LO: nanodisc complex (1:1 ratio) in a lane where only the complex with a ratio of 2:1 was expected, leading to a conclusion that there could be an equilibrium in the binding events.

The enzymatic activity of monomeric 5LO (with and without calcium) was analysed by UV-spectrometry and LC-MS/MS, where the former one was used to measure the formation of 5(*S*)-HPETE and the later one for LTA_4. The data obtained indicated that association keeps the 5LO active and that it is comparable to previous studies with liposomes [81]. Without calcium, the activity of 5LO was reduced considerably, even in the presence of membrane environment. It was almost double in the presence of nanodiscs compared to liposomes. This could be due to sequestering of AA in the liposome interior, which leads to less free AA in the solution, whereas nanodiscs lack this kind of sequestering capacity leading to higher concentration of free AA in solution.

4.1.3 Analysis of dimeric 5LO

The dimeric 5LO did not bind to nanodiscs in the presence of calcium. This could be due to the head to tail dimer configuration of 5LO [43]. This configuration might mask and create a steric hindrance of W75 thereby preventing its association to the membrane. Furthermore, a lack of enzymatic activity is seen with the dimeric 5LO. There is no direct way to assign a lack of activity to dimerisation of 5LO since, discriminating the lack of activity between enzymatic inactivation [108], non-enzymatic inactivation [106] or due to oligomerisation is not possible. Understanding the different mechanisms of inactivation in 5LO could provide more information on regulation of the 5LO.

4.1.4 A method to visualize small membrane binding proteins by TEM

A method was developed to study small membrane interacting proteins like 5LO by TEM and nanodiscs. We took advantage of the aggregation phenomenon of phospholipid liposomes induced by a particular negative stain called sodium phosphotungstate (NaPT). This binding of liposomes on each other leading to so called stacking is probably due to an electrostatic interaction between the phospholipids and the phosphoryl group of phosphotungstate [109]. Since the nanodisc has a lipid bilayer core, a similar effect (Figure 9A) was seen on staining nanodiscs with NaPT. We speculated in our hypothesis (Figure 9B) that since this stacking is caused by electrostatic interaction, any object which may sit in any of the two planar sides of the nanodisc could prevent stacking which could be concluded as a positive interaction with nanodiscs, in other words, the protein is interacting with a membrane.

5LO which binds to nanodiscs in the presence of calcium was used to test this hypothesis. Several combinations - nanodiscs, nanodiscs with calcium, nanodiscs with monomeric 5LO without calcium, nanodiscs with monomeric 5LO with calcium, dimeric 5LO with nanodiscs, dimeric 5LO with nanodiscs and calcium - were analysed. As per hypothesis, monomeric 5LO in the presence of calcium bound to nanodiscs which caused the steric hindrance leading

to prevention of stacking. There was no stacking visualised with the complex of monomeric 5LO-ND with calcium, corroborating the previous results from native pages.

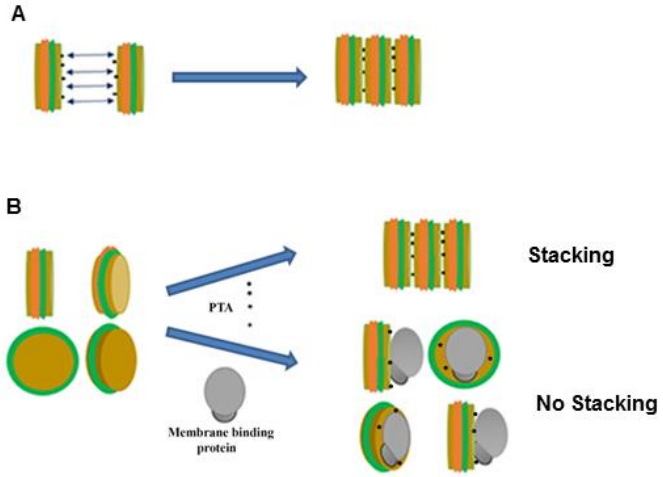

Figure 9: Principle of stacking of nanodiscs

Class averages obtained by TEM from the complex of 5LO-ND showed different interesting binding modes of monomeric 5LO with a membrane. The stacking was seen with the monomeric 5LO with nanodiscs without calcium showing the absence of membrane binding event. Another interesting result was the presence of stacking in the sample containing dimeric 5LO and nanodisc in the presence of calcium. Even if dimeric 5LO can bind calcium ions this does not promote binding to membrane which are in line with the previous native PAGE results. Stacking has also been seen in nanodiscs with calcium indicating that prevention of stacking was not induced by calcium.

4.2 PAPER III

In this paper, the molecular steps leading to the formation of 5-HETE is studied in detail by KIE analysis. The product formation was measured by both UV-spectrometry and RP-HPLC using normal AA and deuterated AA.

4.2.1 Kinetic isotopic effect studies on 5LO

The initiation of leukotriene biosynthesis starts with 5LO oxygenating AA to form 5(*S*)-HPETE followed by a dehydration step to form LTA_4. The pro-S hydrogen is removed from C7 and O_2 is inserted into C5 of AA by 5LO which forms 5(*S*)-HPETE. LTA_4 is formed by removing pro-R hydrogen from C10. To understand the mechanism better, labelled 7,7-d2-AA was compared with AA and HETEs formation was measured at 235nm.

The substrate AA was deuterated which lowered the isotopic effect indicating a change in region-selectivity. Unlabelled AA produced 95% 5-HETE and 8 % 8-HETE whereas labelled AA produced lower 5-HETE (59%) and higher 8-HETE (41%). This shows that selectivity of 5LO has been reduced due to deuteration of AA. The catalytic rate k_{cat} for labelled AA (0.01 s^{-1}) was approximately seven times less than AA (0.06 s^{-1}) which shows decreased deuterium abstraction at C7 together with higher substrate inhibition.

Strong substrate inhibition at higher substrate concentration reduced the KIE leading to a conclusion that functional integrity of 5LO rather than the labelling of the substrate is in play. Kinetic parameters were calculated at low AA concentration leading to less a substrate inhibition. There is a 2 fold change in K_M between the labelled and unlabelled indicating KIE is not affected by substrate binding at low concentration. Altogether it can be safe to infer that the observed KIE for the k_{cat}/K_M is from the hydrogen abstraction step. Arrhenius plot analysis shows a possibility of tunnelling during hydrogen transfer step.

Analysing the structure of 5LO reveals that larger residues like F421, Q363 and L368 might interfere in the AA rearrangement during the catalysis leading to lower reaction rate and also reduces the substrate selectivity leading to the formation of 8-HETE (in the case of labelled AA).

4.3 PAPER IV

The aim of this paper IV was to provide a structure based explanation for the different mechanisms of product formation by the *Xenopus laevis* LTA_4 hydrolase compared to the human LTA_4 hydrolase (see section 1.2.4 and 1.3.2). The mechanism of product formation by $xlLTA_4H$ was studied by spectrometry, RP-HPLC and *in silico* conformer analysis and the structure was solved by X-ray crystallography and SAXS.

4.3.1 Activity assay

The mutant F375Y cannot produce the second product Δ^6-trans-Δ^8-cis-LTB_4 and the catalytic activity of $xlLTA_4H$ is 20 times higher than human. K_M for wt $xlLTA_4H$ 45 µM is higher than K_M for mutant which is 4 µM, this indicates the phenylalanine´s role in binding and recognition of the LTA_4. Substrate selectivity of $xlLTA_4H$ is likely reduced in wildtype due to rapid turnover rate. All the evidence indicates that LTA_4 binds to Y378 in $hLTA_4H$ during catalysis which leads to suicidal inactivation.

With the methyl ester of LTA_4 (LTA_4-ME), the epoxide hydrolase activity is reduced by 20% in wild type and to 80% in mutant F375Y. In the case of aminopeptidase activity, wild type showed 15% reduction in activity and the mutant showed 90% reduction. The fact the activity is reduced in wild type enzyme can be reasoned with earlier studies on human LTA_4H, showed the covalent interaction between Tyr 383 and LTA_4. Testing the wild type and mutant for the second product Δ6-trans-Δ8-cis-LTB_4, indicated that the mutant cannot produce any Δ6-trans-Δ8-cis-LTB_4 and wild type produced Δ6-trans-Δ8-cis-LTB_4 and LTB_4 in the ratio of 1:10. The wild type also produced 5(*S*), 6-DHETE and 5(*S*), 12 DHETE.

4.3.2 Crystallization of $xlLTA_4H$

Limited proteolysis yielded crystals which were used to solve the crystal structure of the $xlLTA_4H$ to 2.3Å. The crystal structure revealed a three domain architecture with the active site in the interface between domains. The striking difference between the human and Xenopus enzyme is the change in residue from tyrosine to phenylalanine at 375. The crystal structure revealed that the active site is narrow for LTA_4 and will cause steric hindrance to form the second product Δ6-trans-Δ8-cis-LTB_4, which requires propeller like movement of LTA_4 chains. LTA_4 cannot change the conformation inside the active site to form the second product.

4.3.3 Conformer analysis

The conformer analysis of LTA_4 revealed that 87% of LTA_4 population in a given system is in S-trans configuration and 6.4% as trans-cis-trans, leading to a conclusion that the former produces LTB_4 and the later one produces Δ6-trans-Δ8-cis-LTB_4. All data indicates that LTA_4H from *Xenopus laevis* produces the second product due to the presence of the conformer already in the solution or it is formed during the entrance of the active site. The phenylalanine at position 375 is less sensitive to the small change in substrate LTA_4 and LTA_4 conformers which may lead to dual product formation in lower organisms.

5 CONCLUSION & FUTURE WORKS

Membrane proteins are notoriously difficult to handle due to their dual nature having both hydrophobic and hydrophilic surfaces. Recent developments in the field of membrane/associated protein handling like amphipols [110], SMALPS [111] and Salipro [112] led to the understanding of proteins through biochemical and biophysical analysis or by structure determination which was not possible before. One of the successful platforms, the nanodisc, makes it possible to provide close to *in vivo* environment *in vitro* [105]. Nanodiscs have been used as membrane mimics to study the enzymatic activity of 15LO [113].

Nanodiscs were used in this thesis instead of other membrane mimics for the following reasons. Detergent micelles would not provide the crucial biological support provided by the lipid membrane surface we needed for the project at hand (5LO does not need FLAP to be present for binding on a membrane surface, only calcium-ions [48,114,115]). Liposomes are better membrane mimics than detergent. Nevertheless, it would be very difficult to visualize the same sample of a protein-membrane-complex by both native PAGE and TEM which were mainly used in this thesis [45,116]. Liposomes can be made of a small size, however, the curvature increases which may be a disadvantage. The advantage of the nanodisc was the size which was carefully chosen.

The nanodiscs used in this thesis were made from MSP1E3D1 that would provide an inner bilayer diameter of 10.5 nm which is an area sufficient for binding one 5LO, an almost cylinder shaped protein with a size of 4.5 nm diameter and 9 nm in length [40]. This size of a disc would also allow the inclusion of one FLAP which is about 4 nm in diameter for the make of a complex with one 5LO and one FLAP. Even though nanodiscs have several advantages mentioned earlier (section 1.5.3), there are some drawbacks like desorption of lipids which would be an issue, being 20 times faster than for liposomes, and should be considered [117]. However, this thesis illustrates nanodisc bilayers has a similar effect as the liposomes in activation of 5LO and showing similar product profile.

Leukotriene biosynthesis starts with the catalytic action of 5LO [48]. Monomeric 5LO is the active form and binds to the membrane surface of nanodiscs in a productive manner in terms of product formation [45]. As mentioned earlier (the above paragraph and in section 1.3.1), 5LO requires another protein called FLAP for efficient catalysis. That the integral membrane protein FLAP and 5LO interact physically has been indicated by several studies *in vivo* [68,118,119]. But how this interaction occurs at the membrane is still unknown, how the presentation of AA to 5LO by FLAP is made and how the activation of 5LO by FLAP occurs are also unknown. Reconstituting FLAP into nanodiscs and analysing its interaction with 5LO through biochemical and structural studies would decipher this interaction. The results would be of importance in understanding the leukotriene pathway and also in the development of new drugs targeting this pathway.

Dimerization of lipoxygenases is caused by several reasons; ligand induces the formation of dimeric 12/15LO [120], dimerization of 12/15LO is due to high salt and higher protein

concentration [121], oligomerization of 11*R*LO is calcium dependent [122]. Dimerization of 5LO has been reported in 2011 [43,45]. Formation of dimeric 5LO is an interesting phenomenon which could be a regulating mechanism to inactivate 5LO in cells. Even though our work corroborates previous works on dimeric 5LO, lack of activity is still not clear. Analyzing the enzymatic activity of dimeric 5LO in cells is the crucial path in understanding the existence of a dimer in the bigger picture of leukotriene biosynthesis.

The endonuclease denoted dicer is a ribonuclease III enzyme involved in the production of microRNAs (miRNAs). It is a multi-domain enzyme and the C-terminus of human dicer has been shown to interact with 5LO through yeast two hybrid system [123]. The molecular details of this interaction would provide a possible role of 5LO in other pathways.

The role of phenylalanine is crucial in LTA_4H in *Xenopus laevis* and is less discriminative on its conformers of the substrates [84]. Analysing the distribution of conformers and altering its distribution may have a profound effect on the activity of LTA_4H. Studying this effect could lead to effectively design drugs against LTA_4H.

The TEM method to visualize small membrane binding proteins can also be used to investigate an interaction between a soluble protein and transmembrane proteins reconstituted into nanodiscs, that is, if the transmembrane protein does not have large extra membranous domains. The simple protocol provides a recipe for the preparation of large quantities of empty nanodiscs which can be used to screen for conditions which could promote peripheral membrane protein binding. In fact, it could be used to screen for conditions where a drug candidate prevents the peripheral protein binding visualized as massive stacking of the nanodiscs used.

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தெய்வத்தான் ஆகா தெனினும் முயற்சிதன்

மெய்வருத்தக் கூலி தரும் (619)

The enthusiasm that I constantly received has been instrumental in my growth and thanks for always supporting me with my endeavors. To my little brother "**Pappu**" and now you can read what I have been doing all these years.

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I am in love with a baby, I am yet to meet. Every step I take is for you. You are my universe!

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