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# **MECHANISM OF ENZYMES INVOLVED IN LEUKOTRIENE C<sub>4</sub> BIOSYNTHESIS**

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Mechanism of Enzymes Involved in Leukotriene C<sub>4</sub>  
Biosynthesis  
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my family*

বাবা, মা, শিরিন, উমার ও সাইফান কে.....



## ABSTRACT

Cysteinyl leukotrienes (cys-LTs) are potent proinflammatory mediators associated with various diseases including asthma and allergic rhinitis. Leukotriene C<sub>4</sub> synthase (LTC<sub>4</sub>S) and microsomal glutathione transferase 2 (MGST2) catalyze conjugation of the epoxide intermediate LTA<sub>4</sub> with GSH to form LTC<sub>4</sub>, the parent compound of the cys-LTs. Both enzymes belong to the Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) super family of integral membrane proteins involved in the generation of lipid mediators and in the metabolism of xenobiotics. This thesis investigates the catalytic mechanism and regulation of LTC<sub>4</sub>S and MGST2. MGST2 can also catalyze conjugation of glutathione (GSH) with electrophilic substrates, such as 1-chloro-2,4-dinitrobenzene (CDNB) and also possesses GSH-dependent peroxidase activity. In this thesis, the overall catalytic mechanism and substrate specificity of human MGST2 has been characterized using purified enzyme. MGST2 ( $k_{\text{cat}}/K_m^{\text{LTA}_4} = 1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) was found to be about 50 times less efficient in catalyzing the biosynthesis of LTC<sub>4</sub> compared to LTC<sub>4</sub>S ( $k_{\text{cat}}/K_m^{\text{LTA}_4} = 8.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ), while the  $K_m^{\text{LTA}_4}$  for MGST2 (40  $\mu\text{M}$ ) and LTC<sub>4</sub>S (30  $\mu\text{M}$ ) were in a similar range. A comparison of LTC<sub>4</sub>S activity with other GSTs suggests that MGST2 could catalyze conjugation of LTA<sub>4</sub> with GSH to form LTC<sub>4</sub> under physiological conditions. Both LTC<sub>4</sub>S and MGST2 bind GSH and activate it to form thiolate anion (GS<sup>-</sup>) at physiological pH and the p*K*<sub>a</sub> of enzyme bound GSH was found to be 5.9 and 6.3, respectively. The mechanism of GS<sup>-</sup> formation was characterized for both enzymes using pre-steady-state kinetics. The amplitude analysis of the signals from all different kinetic and spectroscopic experiments suggested that the GS<sup>-</sup> / enzyme subunit stoichiometry was 3/3 for LTC<sub>4</sub>S and 1/3 for MGST2, which may partly explain the difference in catalytic efficiency. To conclusively show that MGST2 is a functional homo-trimer with one-third-of-the-sites reactivity we combined the results from blue native PAGE, differential scanning calorimetry, isothermal titration calorimetry and equilibrium dialysis followed by global kinetic simulations.

Analysis of all microscopic rates and equilibrium constants for GSH binding and activation suggest that GS<sup>-</sup> formation is not a rate-limiting factor for LTC<sub>4</sub>S, as has been observed for other MAPEG members, such as MGST1. Conversely, GS<sup>-</sup> formation ( $k_2 = 41.1 \text{ s}^{-1}$ ) was faster for MGST2 relative to MGST1, but within only one site of the homo-trimer at a given time. Furthermore, pre-steady-state kinetics using CDNB as an electrophilic substrate showed that the chemical conjugation step is most likely rate limiting for MGST2 catalysis under physiological conditions.

Recently, a ribosomal S6 kinase (p70S6k) was shown to play a role in the phosphoregulation of LTC4S in monocytes. Here, we identified a major p70S6k phosphorylation site on LTC4S as Ser-36, along with a low-frequency site at Thr-40, by an *in vitro* phosphorylation assay followed by mass spectrometric analysis. Phosphomimetic mutants were generated to study the functional consequences of phosphorylation by kinetic analysis, molecular dynamics simulations and structural studies. Our results identified Ser-36 as the functionally important site for the regulation of LTC4S activity, where phosphorylation impairs catalytic activity via a mechanism of hydrogen bonding interactions between the phosphoserine and the catalytically important Arg-104, as well as by limiting substrate access to the active site.

In summary, MGST2 displays broad substrate specificity similar to MGST1, whereas LTC4S is highly specific towards LTA<sub>4</sub> as a physiological substrate. The distinct catalytic and mechanistic properties of MGST2 and LTC4S suggest that while the former may fulfill a promiscuous role in several biochemical pathways, the latter has evolved to fulfill a specific physiological function of LTC<sub>4</sub> synthesis. In general, the acquired knowledge about the LTC4S and MGST2 will be useful for the development of pharmaceuticals against inflammatory diseases, and in addition, will provide context during the physiological and mechanistic characterization of other MAPEG members. Moreover, the observed regulation of LTC4S activity through phosphorylation is unique among the MAPEG members and the results presented herein will provide important clues for understanding the mechanism of phosphoregulation during cys-LT biosynthesis.

## LIST OF SCIENTIFIC PAPERS

- I. **Pre-Steady-State Kinetic Characterization of Thiolate Anion Formation in Human Leukotriene C<sub>4</sub> Synthase**  
Agnes Rinaldo-Matthis, **Shabbir Ahmad**, Anders Wetterholm, Peter Lachmann, Ralf Morgenstern, and Jesper Z. Haeggström.  
*Biochemistry*, 2012, 51, 848–856.
- II. **Catalytic Characterization of Human Microsomal Glutathione S-Transferase 2: Identification of Rate-Limiting Steps**  
**Shabbir Ahmad**, Damian Niegowski, Anders Wetterholm, Jesper Z. Haeggström, Ralf Morgenstern, and Agnes Rinaldo-Matthis.  
*Biochemistry*, 2013, 52, 1755-1764.
- III. **Trimeric microsomal glutathione transferase 2 displays one third of the sites reactivity**  
**Shabbir Ahmad**, Madhuranayaki Thulasingham, Isolde Palombo, Daniel O. Daley, Kenneth A. Johnson, Ralf Morgenstern, Jesper Z. Haeggström, and Agnes Rinaldo-Matthis.  
*Biochimica et Biophysica Acta*, 2015, 1854, 1365-1371.
- IV. **Phosphorylation of Leukotriene C<sub>4</sub> Synthase at Serine 36 Impairs Catalytic Activity**  
**Shabbir Ahmad**, A. Jimmy Yetterberg, Madhuranayaki Thulasingham, Fredrik Tholander, Tomas Bergman, Roman Zubarev, Anders Wetterholm, Agnes Rinaldo-Matthis, and Jesper Z. Haeggström.  
*Journal of Biological Chemistry*, 2016, 291, 18410-18418.

### Additional relevant papers

- Niegowski, D., Kleinschmidt, T., **Ahmad, S.**, Qureshi, A. A., Marback, M., Rinaldo-Matthis, A., and Haeggstrom, J. Z. (2014) Structure and inhibition of mouse leukotriene C<sub>4</sub> synthase. *PLoS One*, 2014, **9**, e96763
- Niegowski, D., Kleinschmidt, T., Olsson, U., **Ahmad, S.**, Rinaldo-Matthis, A., and Haeggstrom, J. Z. (2014) Crystal structures of leukotriene C<sub>4</sub> synthase in complex with product analogs: implications for the enzyme mechanism. *J. Biol. Chem.* **289**, 5199-5207

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## LIST OF ABBREVIATIONS

LT	Leukotriene
PG	Prostaglandin
LA	Linoleic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
LO	Lipoxygenase
GSH	Glutathione
MAPEG	Membrane associated proteins in eicosanoid and glutathione metabolism
mPGES1	Microsomal prostaglandin E synthase 1
FLAP	Five lipoxygenase activating protein
LTC <sub>4</sub> S	Leukotriene C <sub>4</sub> synthase
MGST1	Microsomal glutathione transferase 1
MGST2	Microsomal glutathione transferase 2
MGST3	Microsomal glutathione transferase 3
CDNB	1-Chloro-2,4-dinitro benzene
DDM	n-dodecyl-β-D-maltopyranoside
5-HpETE	5( <i>S</i> )-hydroperoxy-6- <i>trans</i> -8,11,14- <i>cis</i> -eicosatetraenoic acid
LTA <sub>4</sub>	5( <i>S</i> )- <i>trans</i> -5,6-oxido-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTB <sub>4</sub>	5-( <i>S</i> ), 12-( <i>R</i> )-dihydroxy-6, 8,10,14-eicosatetraenoic acid
LTC <sub>4</sub>	5( <i>S</i> )-hydroxy-6( <i>R</i> )- <i>S</i> -glutathionyl-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTD <sub>4</sub>	5( <i>S</i> )-hydroxy-6( <i>R</i> )- <i>S</i> -cysteinylglycyl-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTE <sub>4</sub>	5( <i>S</i> )-hydroxy-6( <i>R</i> )- <i>S</i> -cysteinyl-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
Cys-LTs	Cysteinyl leukotrienes
p70S6k	Ribosomal S6 kinase
GST	Glutathione transferase
GS <sup>-</sup>	Thiolate anion
GSO <sub>3</sub> <sup>-</sup>	Glutathione sulphonic acid



# 1 INTRODUCTION

## 1.1 LIPIDS IN CELLULAR CONTEXT

Water, inorganic ions and carbon containing organic molecules are the major constituents of cells. There are four classes of organic molecules that are important as cell constituents; they are carbohydrates, lipids, proteins and nucleic acids. Among these organic molecules lipids are one of the important components of cells. Lipids are playing three major roles in cells; they constitute the cell membrane, as a form of energy storage, and play important roles as signaling molecules. Generally, lipids are fatty acid molecules containing a long hydrocarbon chain with a carboxyl group ( $\text{COO}^-$ ) at one end. This long hydrocarbon chain is responsible for its hydrophobic nature. Lipids are stored within the cell in the form of triacylglycerol. Triacylglycerols are insoluble in water as they contain three fatty acids attached to a glycerol molecule. Triacylglycerols accumulate as fat droplets in the cytoplasm and break down through energy yielding reactions.

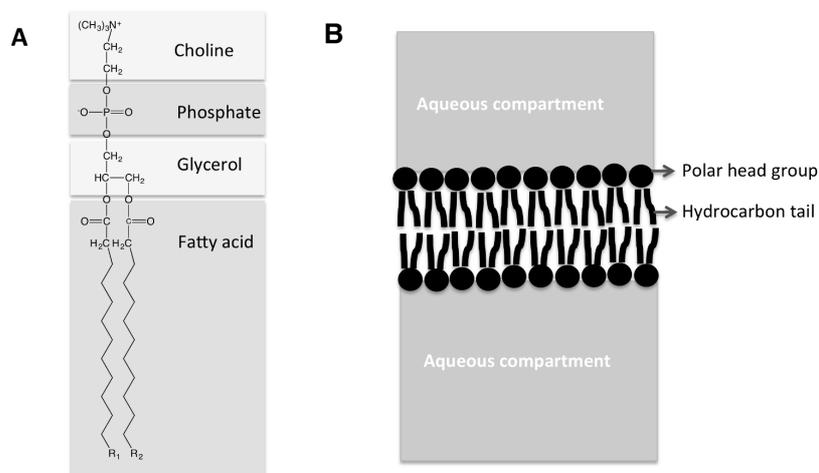


Figure 1: (A) Chemical structure of phosphatidylcholine, one of the major constituents of biological membranes. (B) A general form of phospholipid bilayer.

Phospholipids are one of the important forms of lipids, known as the principal components of all cell membranes. They are amphipathic molecules, which contain two fatty acid chains linked to a phosphate containing polar head group (Figure 1A). Because of their amphipathic nature, phospholipids spontaneously form bilayers in aqueous environments. In the bilayers, the hydrocarbon tails are buried inside because of their hydrophobic nature. The polar head

groups are exposed to the aqueous solutions on both sides (Figure 1B). This type of phospholipid bilayers acts as a stable barrier between two aqueous compartments within the cells, which are the basic form of all biological membranes. Sphingomyelin is another class of membrane lipids composed of sphingosine, a fatty acid, a phosphate group and choline. Molecules like ceramide and sphingosine are derived from sphingomyelin by sequential cleavage. In addition to phospholipids and sphingomyelins, biological membranes also contain glycolipids and cholesterol in eukaryotic cells. Cholesterol sits within the gap in the middle of sphingomyelin molecules (1). The presence of sphingomyelins and cholesterol at high concentrations are found in lipid raft regions. Many signaling proteins have been found to reside or transport through the raft during signaling processes (2). Lipid raft regions are also known for anchoring extracellular proteins with glycosylphosphatidylinositol and associating intracellular or transmembrane proteins (3). Fatty acyl chains attached to membrane lipids may contain one or more double bonds. The presence of double bonds in the hydrocarbon chain introduces kinks in the structure, which occupy more space. The unsaturated fatty acids, thus, make membrane more fluid (1).

## **1.2 LIPID MEDIATORS**

Apart from constituting the cellular membrane, lipids play key roles in various physiological as well as pathophysiological signaling processes to pertain cellular functions such as, cell proliferation, apoptosis, metabolism and migration (4). Usually, hydrolysis of membrane lipids generates a wide variety of fatty acids. Fatty acid molecules are involved in many cellular processes by acting as messenger molecules in signaling pathways. Fatty acid signaling molecules are often associated with pathophysiological conditions like inflammation, cancer and metabolic disease (4). The focus of this thesis is on the lipid mediators involved in inflammatory processes. Inflammation is a process initiated by our body's defense mechanism in response to different types of physical and microbial insults like injury or viral/bacterial infections. This process most often causes both beneficial and harmful effects for our health. The inflammatory process helps to protect our body against foreign microorganisms as well as to start healing processes to any kind of tissue injury. On the other hand, it also causes diseases when inflammatory mediators formed in excess in our cellular environment. Inflammatory processes consist of a wide range of physiological and pathological responses coordinated by a variety of lipid mediators (5). Eicosanoids is one of the most well-known classes of lipid mediators, which consists of hydroperoxyeicosatetraenoic acids (HpETE), prostaglandins (PG), thromboxanes (TX),

leukotrienes (LT) and Lipoxins (LX). They all are derived from a polyunsaturated fatty acid named arachidonic acid (AA), which is usually found esterified at the sn2 position of glycerophospholipids in the membrane bilayer and released by enzymatic hydrolysis. The intracellular arachidonic acid is then metabolized to different classes of eicosanoids through several enzymatic pathways (Figure 2). The eicosanoids are usually pro-inflammatory (6). In contrast, lipoxins acts as pro-resolving molecules (7) and PGE<sub>2</sub> displays both pro and anti-inflammatory effects (8). There are also other important classes of lipid mediators generated from different precursor fatty acid molecules such as linoleic acid (LA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) through enzymatic oxidations (9). The mediators derived from LA can also act as both pro and anti-inflammatory effector molecules (10). The ω-3 polyunsaturated fatty acids, such as EPA and DHA generate anti-inflammatory or pro-resolving lipid mediators by enzymatic oxygenation, and comprise resolvins, protectins, and maresins (7,8).

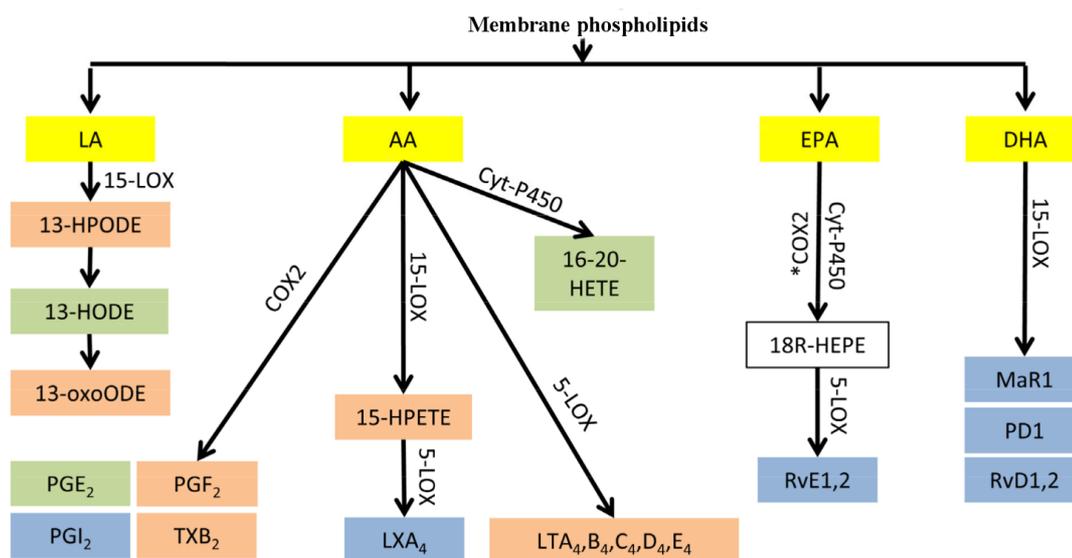


Figure 2: Different class of lipid mediators generated through enzymatic oxidation from their respective precursor molecules (Marked yellow). The pro-inflammatory mediators are shown in orange boxes while resolving molecules are in blue boxes. The mediators with variable functions are shown in green boxes. The enzymes involved in the biosynthesis of those lipid mediators are shown in the respective pathways such as lipoxygenases (LOX), cyclooxygenases (COX), aspirin acetylated cyclooxygenase 2 (\*COX2) and cytochrome-P450 complex (Cyt-P450). The lipid mediators are hydroxyoctadecadienoic acid (HODE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosapentaenoic acid (HEPE), leukotrienes (LT), lipoxins (LX), maresins (MaR), prostaglandins (PG), resolvins (Rv), protectins (PD) and thromboxanes (TX). Figure adopted from (9).

The biosynthesis of these lipid mediators are of great interest as the corresponding enzymes can be overexpressed in the respective inflammatory cells upon stimulation. On the other hand, the same lipid mediators can also be formed through non-enzymatic autoxidation by reactive oxygen species and contribute to the pathophysiology of many diseases such as, atherosclerosis, asthma, Alzheimer's and Parkinson's disease (11). Several of these lipid mediators involved in the inflammatory processes are formed from their parent molecule through conjugation with a tripeptide, glutathione.

### 1.3 GLUTATHIONE METABOLISM

Glutathione (GSH),  $\gamma$ -glutamylcysteinylglycine, is a small tripeptide molecule containing a cysteinyl thiol, which acts as a nucleophile. GSH, with its nucleophilic thiol, plays an important role in detoxifying the reactive electrophilic intermediates and metabolically produced oxidizing agents (12). The  $\gamma$ -glutamyl peptide bond in GSH provides intracellular stability by preventing it from proteolysis by peptidases (13). GSH is a non-protein thiol and is abundant (in millimolar range) in most mammalian cells (13,14).

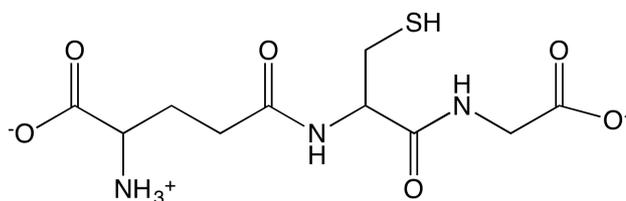
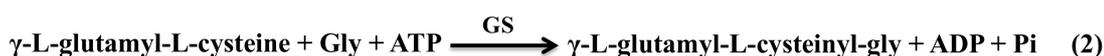


Figure 3: Chemical structure of glutathione.

Glutathione is metabolized through different enzymatic and non-enzymatic reactions within the cell by various intracellular and transcellular pathways. The well-established cellular use of GSH is to form conjugates with endogenous and xenobiotic compounds to protect cells against toxicants (15). This type of reaction is usually catalyzed by glutathione transferases where GSH is consumed as a substrate. The GSH conjugated molecules are usually excreted from cells and the overall hydrophilicity of the GSH molecule helps to attain aqueous solubility of such conjugates (12). GSH is utilized as a cofactor or consumed during the biosynthesis of several eicosanoids, such as prostaglandins and leukotrienes. It is used as a

reductant by glutathione peroxidases to catalyze peroxidase reactions towards hydroperoxides and lipid peroxides to generate corresponding alcohol (ROH) and glutathione disulfide (GSSG) and regulate the intracellular redox state. GSH can also interact with glutaredoxin and protein disulfide isomerases, and play complementary roles together with thioredoxin in cytoprotection (15). In addition, it can serve as a storage and transport form of cysteine (16).

As GSH is consumed through different metabolic reactions, it is important to replenish with new molecules to maintain intracellular GSH concentrations. The regeneration of GSH mainly occurs by *de novo* synthesis in two enzymatic steps. In the first step, an enzyme named glutamate cysteine ligase (GCL, E.C. 6.3.2.2) is involved to produce  $\gamma$ -glutamylcysteine from its constituent amino acids (Reaction 1) and in the following step; glutathione synthase (GS, E.C.6.3.2.3) catalyzes the addition of glycine to  $\gamma$ -glutamylcysteine to form GSH (Reaction 2). Both enzymes are ATP dependent (15).



Another way of regeneration is by recycling from GSSG, which is reduced back to GSH by the action of glutathione reductase (GR, EC 1.6.4.2) at the expense of NADPH (Reaction 3).



Therefore, balanced GSH metabolism is essential for both cellular defense mechanism and normal physiology (15).

#### 1.4 MAPEG SUPER FAMILY

Mammalian glutathione transferases are divided in three classes; cytosolic, mitochondrial and membrane bound glutathione transferases (17). Membrane bound glutathione transferases are integral membrane proteins belonging to the MAPEG superfamily. Membrane Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) family consists of six human transmembrane proteins. The name “MAPEG” was first designated based on their enzymatic activities, sequence motifs and structural properties (18). They are microsomal prostaglandin E synthase 1 (mPGES1), five lipoxygenase activating protein (FLAP), leukotriene C<sub>4</sub> synthase (LTC4S), microsomal glutathione transferase 1 (MGST1), microsomal glutathione transferase 2 (MGST2) and microsomal glutathione transferase 3 (MGST3). Structure based

sequence alignment of the six human MAPEG members indicates the clustering into three clans, where LTC4S, MGST2 and MGST3 form one clan while MPGES1 and MGST1 cluster into another clan (19). The most common features among these MAPEG family members are that they all use glutathione as a co-factor for catalysis, form homotrimers, and are located in the endoplasmic reticulum (ER) or nuclear membrane. Based on their membrane localization, one can speculate that physiological substrates may be derived from membrane based lipid molecules or from the products of lipid peroxidation.

#### **1.4.1 MGST1**

Microsomal glutathione transferase 1 (MGST1, EC 2.5.1.18) was first isolated and characterized from rat liver microsomes (20-23). MGST1 displays broad substrate specificity as cytosolic glutathione transferases (GST) and upon activation increases its catalytic efficiency significantly (24). The enzyme catalyzes the conjugation reaction between GSH and reactive electrophilic substrates such as halogenated aromatic hydrocarbons, and phospholipid hydroperoxides (25-27). It thus also possesses GSH dependent peroxidase activity (28). MGST1 is highly abundant in the endoplasmic reticulum of rat and human liver (28,29) and in the outer mitochondrial membrane in the rat liver (30). As this enzyme is involved in the biotransformation of lipophilic reactive intermediates and the reduction of lipid hydroperoxides, it is speculated that MGST1 is involved in the protection of membranes from lipid peroxidation (31) and cells from oxidative stress (32). The enzyme is also involved in drug metabolism by conjugating xenobiotic compounds with GSH (33). MGST1 is a 17 kDa homo-trimeric enzyme and its crystal structure was determined by electron crystallography (34), which came out to be the first known structure among the MAPEG members at that time. The GSH binding site is found within the monomer interface with an extended conformation (34). MGST1 has been extensively studied in terms of catalytic mechanism (35-38). It displays a bimolecular random sequential mechanism (35) and the activated enzyme shows up to 30-fold increased activity compared to unactivated enzyme. The ability of this enzyme to be activated is due to the modification by sulfhydryl reagents (20,21). GSH binding and activation is essential for its catalytic mechanism like other glutathione transferases and thiolate anion formation contributes to the turn over for both activated and unactivated enzyme (37). The residue involved in the stabilization of the thiolate anion has not been experimentally proven but it is speculated that Arg-129 is the one involved in this process based on the results from other MAPEG members (24). MGST1 exhibits one-third-of-the-sites reactivity towards GSH, which indicates that GSH binding

occurs with different affinity at all three active sites (39). MGST1 has now become an interesting anti-cancer drug target as it can protect tumors from cytostatic drug treatment (40).

#### **1.4.2 mPGES1**

Microsomal prostaglandin E synthase 1 (mPGES1) is one of the terminal enzymes in the prostaglandin biosynthesis pathway, previously named as MGST1-L1 as it shows 38% sequence similarity with MGST1. Later, it was identified as microsomal glutathione-dependent PGE synthase (41). It catalyzes the isomerization of endoperoxide PGH<sub>2</sub> into PGE<sub>2</sub>. Apart from the PGE synthase activity, mPGES-1 can catalyze the oxidoreduction of endocannabinoids to prostaglandin glycerol esters (42) and PGG<sub>2</sub> into 15-hydroperoxy-PGE<sub>2</sub> (43). In addition, mPGES1 displays glutathione dependent peroxidase activity and is able to conjugate 1-chloro-2,4-dinitro benzene (CDNB) with glutathione (GSH) as a glutathione transferase, but with very low activity (43). It has become a potential therapeutic target as mPGES-1 generated PGE<sub>2</sub> plays key roles in several pathological conditions like inflammation, pain, fever, anorexia, atherosclerosis, stroke, and tumorigenesis (44). The 3D crystal structure of mPGES-1 was determined by electron crystallography from 2D crystals (45) and recently, by X-ray crystallography at 1.2 Å resolution (46) in complex with GSH. The binding of GSH is observed as U-shaped in both structures. In addition, a cytosolic domain named as the C domain, was identified in the recent structure. The C domain forms part of the active site and is a novel structural feature observed among MAPEG members. The crystal structure reveals that mPGES-1 forms a homotrimer with three active sites within the monomeric interfaces. Like other MAPEG members, GSH thiolate anion formation is suggested to be important for catalysis, where Arg-126 is involved in the stabilization of the thiolate anion after breaking its critical and dynamic interaction with Asp-49 (47). The mPGES-1 displays one-third-of-the-sites reactivity (48), which is common among several MAPEG members, such as MGST1 (39).

#### **1.4.3 FLAP**

Five lipoxygenase activating protein (FLAP) is another important MAPEG member involved in arachidonic acid (AA) metabolism through the lipoxygenase pathway (49,50). Although it shows significant degree of structural similarity with other MAPEG members, it does not have any enzymatic activity. However, FLAP is believed to play an important role in delivering AA as a substrate to 5-lipoxygenase for further metabolism (51). The crystal

structure of FLAP was determined at 4.0 Å and 4.2 Å resolution by X-ray crystallography in complex with an inhibitor MK-591 and an iodinated analog of MK-591 respectively. The FLAP inhibitors block the transfer of AA to 5-LO by binding to the same site, located within the membrane-spanning region where AA can laterally diffuse from membrane bi-layer to the FLAP (52).

#### 1.4.4 LTC<sub>4</sub>S

Leukotriene C<sub>4</sub> synthase (LTC<sub>4</sub>S, EC 4.4.1.20) is an integral membrane protein distinct from other cytosolic and microsomal GST's, because of its narrow substrate specificity and inability to conjugate GSH with xenobiotics (53). The enzyme catalyzes the formation of leukotriene (LT) C<sub>4</sub> from an allylic epoxide intermediate, leukotriene (LT) A<sub>4</sub>, by conjugating it with a GSH molecule. The conjugation of LTA<sub>4</sub> with GSH to form LTC<sub>4</sub> was observed in human leukocytes (54), RBL cells (55,56), and rat liver (57). Later, the enzyme responsible for this biotransformation was termed leukotriene C<sub>4</sub> synthase (LTC<sub>4</sub>S). LTC<sub>4</sub>S is expressed and present in a limited number of cell types such as eosinophils, mast cells, basophils, monocytes, macrophages and platelets (58). It has been isolated and purified from different sources, such as rat leukemia cells (59), human monocytic cell lines (60), and guinea pig lung (61). Initially, it was believed that LTC<sub>4</sub>S functions as a homo-dimer when first time purified to homogeneity (62). The structural information first appeared in 2004 when a projection map of LTC<sub>4</sub>S at 4.5 Å resolution was calculated from 2D crystals by electron crystallography, where it was shown that LTC<sub>4</sub>S constitutes a trimer (63). Later in 2007, two research groups simultaneously presented the high-resolution crystal structures of LTC<sub>4</sub>S and concluded that it forms a symmetric homotrimer as a functional unit. One group has presented an apo and a GSH complexed structure of LTC<sub>4</sub>S at 2.0 and 2.15 Å resolution respectively (64) and the other group determined a GSH complexed structure at 3.3 Å resolution (65). Each of the monomers within the trimer consists of four transmembrane  $\alpha$ -helices and GSH is bound within the monomer interface with a horseshoe shaped conformation. The unique conformation of GSH allows the positioning of the thiol group in a way that it can effectively interact with neighboring arginine residues for activation. The data obtained from site-directed mutagenesis and crystal structure identified Arg-104 as a key catalytic residue for thiolate anion stabilization (66). A catalytic mechanism of LTC<sub>4</sub>S was also proposed with the involvement of two arginine residues, Arg-104 and Arg-31 from neighboring monomer, where they believe Arg-31 is involved in the epoxide ring opening of LTA<sub>4</sub> (67). Dodecylmaltoside (DDM), a detergent molecule, is a mimic of the lipid substrate LTA<sub>4</sub> as it

contains similar structural properties like LTA<sub>4</sub> such as amphipathic nature and a long hydrophobic tail. DDM was identified as bound to the active site close to GSH in both apo and GSH complexed structure of LTC<sub>4</sub>S (64), which indicates the location of the lipid substrate-binding site. The difference between the binding modes of the DDM molecule within the apo and GSH complexed structure provides an explanation for the important role of Trp-116, which is believed to form a lid over the ω-end of the lipid substrate to position the hydrophobic tail and proposed that the effective positioning of lipid substrate is facilitated by GSH binding (64). Later, several crystal structures of LTC<sub>4</sub>S were determined in complex with three product analogs to demonstrate a route for product release aided by Trp-116 (68).

LTC<sub>4</sub>S is the only enzyme within the MAPEG super family reported to be regulated through phosphorylation (69-71). The present investigation in this thesis is focused on the catalytic mechanism for thiolate anion formation with pre-steady state kinetics and identifying the mechanistic details of phosphoregulation of LTC<sub>4</sub>S.

#### **1.4.5 MGST2**

Microsomal glutathione transferase 2 (MGST2, EC 2.5.1.18) is a 17 kDa integral membrane protein, first identified and characterized in 1996 with LTC<sub>4</sub>S activity and significant sequence identity to LTC<sub>4</sub>S (44%) (72). According to the evolutionary tree, plotted using the sequence information of MAPEG family proteins from sequence databases and compile genome location and structure, MGST2 is the closest relative to FLAP and LTC<sub>4</sub>S (73).

MGST2 displays wide tissue distribution at mRNA level and is specifically expressed in human liver, spleen, skeletal muscle, heart, adrenals, pancreas, prostate, testis, fetal liver and fetal spleen while low level of mRNA expression was observed in lung, brain, placenta and bone marrow. Based on the mRNA expression profile, MGST2 differs from FLAP as FLAP mRNA is detected in lung, various organs in the immune system and in leukocytes (72). In another study, it was demonstrated that MGST2 was predominantly expressed in human liver microsomes and endothelial cell membranes while LTC<sub>4</sub>S was found in human lung membranes, platelet homogenates and kidney tissues (74).

MGST2 is known to have LTC<sub>4</sub>S enzyme activity together with glutathione transferase activity as it can catalyze the conjugation of GSH with LTA<sub>4</sub> and 1-chloro-2,4-dinitro benzene (CDNB), indicating overlapping functional roles with other MAPEG members, especially with LTC<sub>4</sub>S and MGST1. It was proposed as a critical enzyme for transcellular

biosynthesis of LTC<sub>4</sub> in the vascular wall when MGST2 was found to be the only enzyme responsible for LTC<sub>4</sub> production in human umbilical vein endothelial cells (75). Beside this LTC<sub>4</sub>S activity, MGST2 displays broad substrate specificity like other glutathione transferases including GSH dependent peroxidase activity (Paper II). As MGST2 is expressed abundantly in human liver and is able to conjugate xenobiotic compounds, it appears to be an important enzyme in the cellular defense system. A most recent study demonstrated that MGST2 is the key enzyme for producing LTC<sub>4</sub> in the cells of non-haematopoietic lineage under endoplasmic reticular (ER) stress (76). However, the original physiological function of this enzyme is yet to be discovered.

It is always challenging to determine a crystal structure for a membrane protein, therefore, precise structural information for this protein is still lacking. On the other hand, it has significant sequence similarities with its homologue LTC<sub>4</sub>S, which make it easy to speculate that MGST2 also forms a homotrimer and binds and activates GSH in a similar fashion as LTC<sub>4</sub>S. A recent study demonstrated that MGST2 functions as a trimer and displays one-third-of-the-sites reactivity like its distant family members MGST1 and mPGES1 (Paper III).

The current thesis is focused on the characterization of MGST2 in terms of substrate specificity and kinetic mechanism in relation with other related MAPEG members.

#### **1.4.6 MGST3**

Microsomal glutathione transferase 3 (MGST3, EC 2.5.1.18) is another integral membrane protein belonging to the MAPEG superfamily. It shares 36% sequence identity with its closely related enzyme MGST2. It was identified and characterized in 1997 and shows similar hydrophobicity pattern like FLAP, LTC<sub>4</sub>S and MGST1 (77). MGST3 also displays a wider tissue distribution as compared to MGST2 and is detected in brain, placenta, liver, pancreas, thyroid, testis, ovary and kidney tissues. Evolutionarily it forms a unique subgroup within the MAPEG superfamily yet possesses LTC<sub>4</sub>S activity and glutathione dependent peroxidase activity like MGST2 (77). It should be noted that, LTC<sub>4</sub>S activity was not observed for rat MGST3 (78). Furthermore, it was reported that plant MGST3 shows a low glutathione transferase activity as it can conjugate CDNB with GSH (73). Moreover, It has recently been proposed that MGST3 is associated with neurodegenerative diseases like Alzheimer's (79).

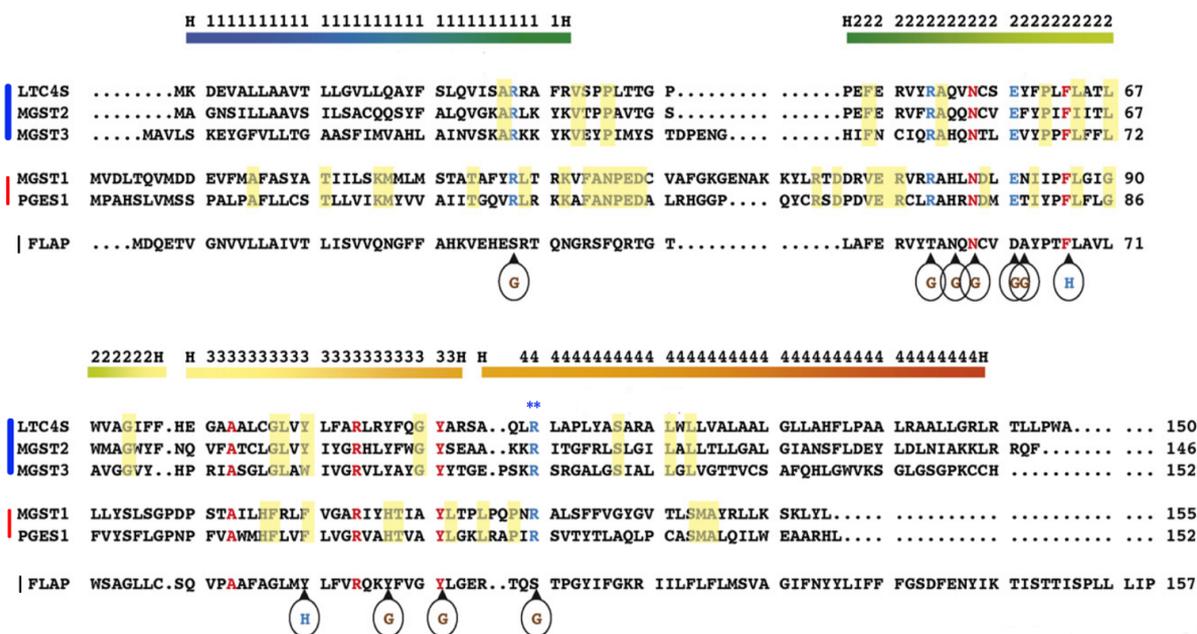


Figure 4: Structure based sequence alignment of MAPEG family proteins. The transmembrane helices are indicated with colored boxes based on the LTC4S structure. There are three clans identified based on the sequence similarity, which are indicated by the vertical blue (clan 1), red (clan 2) and black (clan 3) lines in the left. The amino acids conserved in all MAPEG members are shown in red, while blue color indicates the amino acids conserved between clan 1 and 2. The conserved regions within each clan are highlighted by yellow color. Letter G indicates the residues involved in the GSH binding and letter H indicates the conserved aromatic residues involved in making key hydrophobic core interactions near the substrate-binding region. The catalytically important arginine residue is pointed out by asterisk. Figure adopted from (19).

#### 1.4.7 Common structural features among MAPEG members

Several unique structural features are common among the MAPEG family proteins based on their known structures obtained till now. The four structurally characterized members display symmetric homotrimeric orientation and each of the subunits consist of four transmembrane helices. The arrangements of transmembrane helices within the trimer retain the similar fold. A C-terminal  $\alpha$ -helix was found only in the LTC4S structure while a unique C domain was seen in the loop region connected between helices 1 and 2 in the mPGES1 structure (Figure 5). The C domain may also be present in the MGST1 structure as that region was poorly determined due to low-resolution data from electron crystallography. The N and C-terminal part both reside in the same side of the membrane. The loops, connecting helices 1 and 2 and helices 3 and 4, are found on the other side of the membrane. The predicted membrane topology, based on theoretical calculations on a database (80), indicates that the loop side of

the protein is pointing towards the cytosol. In contrast, the opposite topology was suggested when a combination of immunofluorescence technique together with differential membrane permeabilization were used to determine the topology. The active site containing loop part of the LTC4S protein was shown to be localized towards the luminal side of the endoplasmic reticulum and nuclear envelope (81). The active site is located within the monomer interface where residues from helices 2, 3, 4 from one subunit and helices 1 from the neighboring subunit participate to bind and activate the substrates. A common conserved RXXXNXXE/D motif has been seen in the helices 2, which is believed to be involved in GSH binding (19). The GSH binding residues are usually conserved among the MAPEG members except for FLAP (Figure 4), which may explain its inability to bind GSH and thus has no enzymatic activity. The catalytically important arginine residue used for thiolate anion stabilization is highly conserved among the MAPEG members while FLAP lacks that particular residue (Figure 4). Structures of the three MAPEG members were determined in complex with GSH where a horseshoe shape conformation for GSH was obtained in both LTC4S and mPGES1. GSH is positioned in a way such that the thiol group is facing towards the membrane interface where it can interact with lipid substrates entering from the membrane lipid bilayer. The GSH is found with an extended conformation in the MGST1 structure. This could be due to the fact that it was poorly determined at a low-resolution structure or that GSH adopts different conformations during binding and activation. A lipid substrate-binding site has also been proposed for all MAPEG members in the membrane interface between two adjacent monomers based on the position of a DDM molecule in the LTC4S structure. The inhibitors bound within the FLAP structure occupy some part of the GSH binding site as well as the hydrophobic site at the membrane interface, which indicates the expected location for the lipid substrate-binding site in other MAPEG members.

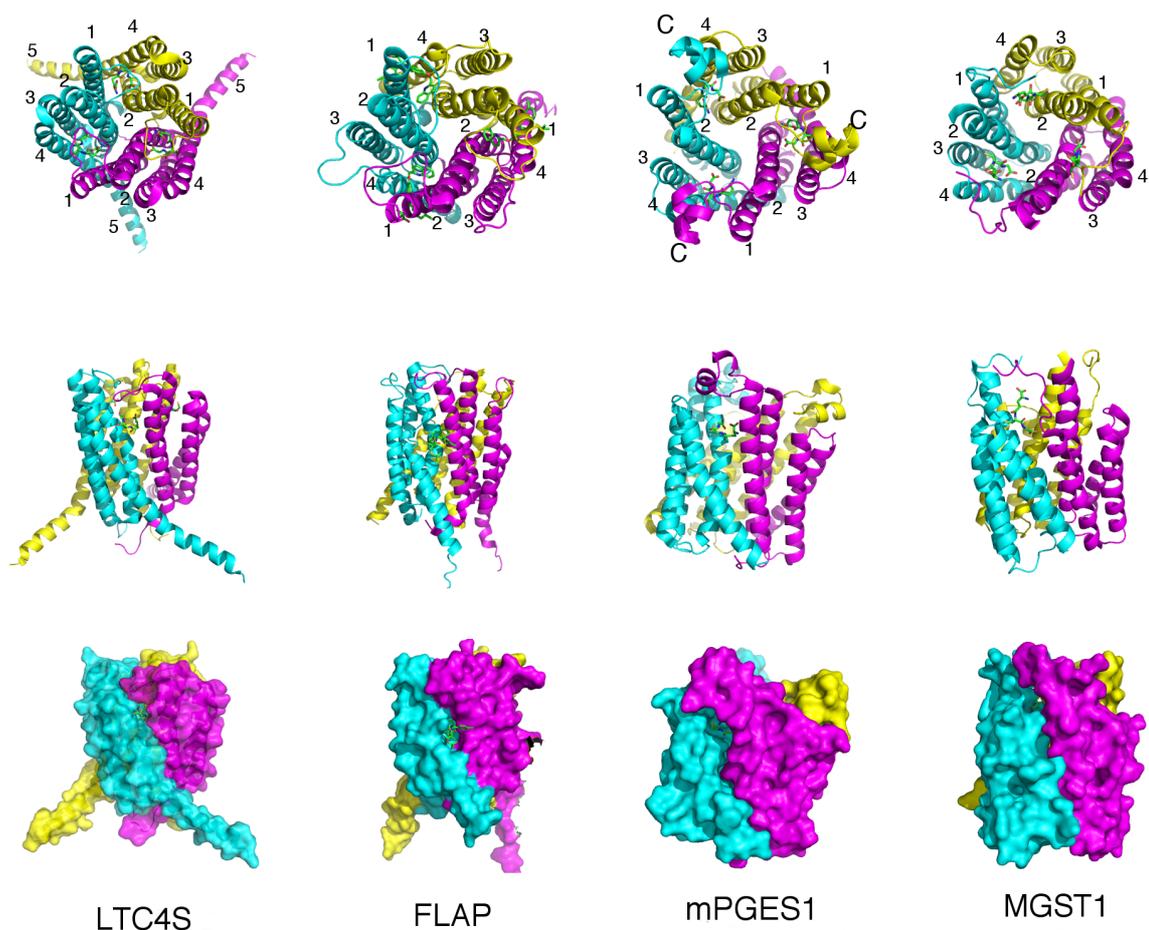


Figure 5: The common trimeric form of MAPEG family proteins. The upper panel is the view from the top to show how the four helices from each subunit participate to form a symmetric homotrimer. The inner core of the trimer is formed by helix 2 from each subunit, which is found to be a common characteristic of all four members. The middle (Cartoon representation) and lower panel (Surface representation) is viewing from the membrane plane to show the active site within the monomer interface. GSH is bound within the deep active site pocket in the structure of LTC4S and mPGES1 while it is located in a slightly different place, above from the deep active site pocket with an extended conformation, in the MGST1 structure. An inhibitor molecule MK-591 is bound at the active site of FLAP, which seems to occupy the same site as GSH and lipid substrates occupy in other structures. The figure is prepared by Pymol using PDB files; 2UUH, 2Q7M, 4AL0 and 2H8A.



## 2 THEORETICAL BACKGROUND

### 2.1 LEUKOTRIENES

Leukotrienes are a family of lipid mediators derived from arachidonic acid (AA) through the lipoxygenase pathway and have potent pro-inflammatory effects. This group of molecules was first recognized while investigating the arachidonic acid metabolism in rabbit polymorphonuclear leukocytes (82). They identified 5-(*S*),12-(*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid as a new metabolite of arachidonic acid, which later was termed leukotriene (LT) B<sub>4</sub>. The unstable epoxide intermediate, 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, formed during the formation of 5,12-dihydroxyeicosatetraenoic acids, was detected later in a following study (83). The epoxide intermediate was then denoted as LTA<sub>4</sub>, a precursor molecule for leukotrienes. LTA<sub>4</sub> biosynthesis begins with an initial formation of 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HpETE) from arachidonic acid, which is then converted into LTA<sub>4</sub> by abstraction of a hydrogen at C-10 followed by the elimination of a hydroxyl from the hydroperoxy group (84). Leukotrienes are separated into two major classes; the dihydroxy acid compound, LTB<sub>4</sub>, represents one class and the other class consists of cysteinyl-leukotrienes (cys-LTs) (85). Initially, cys-LTs were known as slow-reacting substance (SRS) or slow-reacting substance of anaphylaxis (SRS-A). Anaphylaxis is a life-threatening allergic reaction initiated through binding of an antigen to immunoglobulin E and the release of inflammatory mediators (86). The term SRS was given for their role as a smooth muscle contracting factor (87). They are referred to as SRS-A when identified together with histamine as a result of antigen-antibody reaction to create slow and long lasting contraction of guinea-pig intestine and found resistant to anti-histamine drugs (88). SRS has also been shown to be important as an asthma mediator and in hypersensitivity reactions (89). In the beginning, they were reported as low molecular weight polar lipids with a conjugated double bond system (90,91). Another study then described SRS-A as a family of thiol lipids, which are derived from arachidonic acid (92). It was reported that biosynthesis of these substances originate from arachidonic acid and increase upon calcium ionophore treatment (93,94). At this stage, a hypothesis was developed based on the existing structural data and ionophore effect where a biogenetic relation was proposed between the previously identified unstable epoxide intermediate and the SRS-A (84). Then the structural identification of 5(*S*)-hydroxy-6(*R*)-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, LTC<sub>4</sub>, was revealed, which showed that SRS-A are derivatives of 5-hydroxy-7,9,11,14-eicosatetraenoic acid with a

glutathione molecule attached to it at C-6 in a cysteinyl thioether linkage (95,96). Later, two other cys-LTs, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, LTD<sub>4</sub>, and 5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, LTE<sub>4</sub>, were found, which are formed by the sequential cleavage of the glutathione peptide moiety of LTC<sub>4</sub> catalyzed by  $\gamma$ -glutamyl transpeptidase and dipeptidase respectively (85,97,98). Finally, it was proven that SRS-A are a mixture of cysteinyl leukotrienes that consists of three molecules, that is the parent molecule LTC<sub>4</sub>, and its metabolites LTD<sub>4</sub> and LTE<sub>4</sub>. The biosynthetic pathway for these substances was then confirmed by involving two steps. The first step includes the formation of unstable epoxide intermediate, LTA<sub>4</sub>, from arachidonic acid via 5-HPETE and in the second step, conjugation of a GSH molecule with LTA<sub>4</sub> through the ring opening of the epoxide moiety at the allylic carbon C-6 (84).

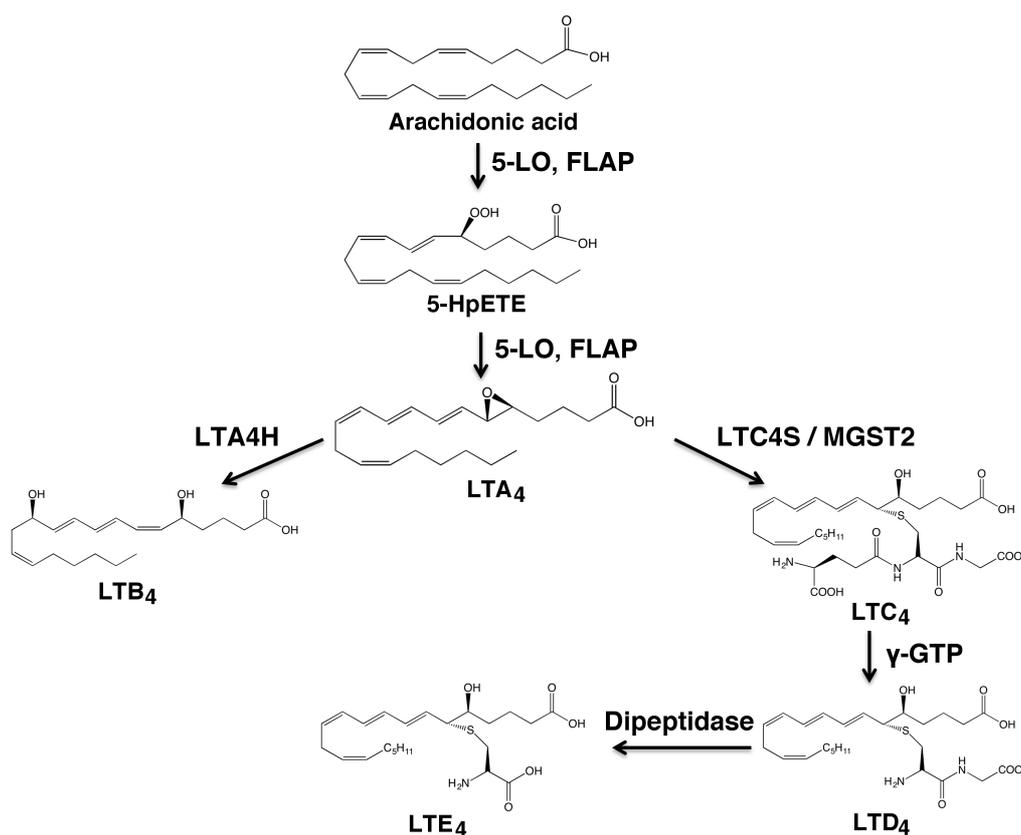


Figure 6: Biosynthesis of leukotrienes from arachidonic acid. 5-LO: 5-lipoxygenase; LTA<sub>4</sub>H: leukotriene A<sub>4</sub> hydrolase;  $\gamma$ -GTP:  $\gamma$ -glutamyl transpeptidase.

The name 'leukotriene' for this family of lipid mediators was given because they were identified from leukocytes and contain a conjugated triene system within their structure. The individual members are designated alphabetically and subscript denotes the number of double bonds present in the structure (99).

Leukotriene biosynthesis occurs in different types of cells, for example, LTB<sub>4</sub> is generally synthesized in neutrophils, macrophages/monocytes, mast cells and in dendritic cells whereas cys-LTs are formed in macrophages/monocytes, eosinophils, basophils, mast cells and in dendritic cells (100). This family of eicosanoids elicits their biological responses through paracrine signaling to the local cellular targets (85).

Leukotrienes are associated with a wide variety of inflammatory diseases. LTB<sub>4</sub> is one of the most potent chemotactic agents known to date and is responsible for the recruitment, activation and survival of leukocytes, such as neutrophils and eosinophils (101-103). Pathophysiological roles for LTB<sub>4</sub> and its receptors have been demonstrated for several diseases such as severe asthma, arthritis, atherosclerosis, chronic obstructive pulmonary disease (COPD), cancer, and in atopic dermatitis (100,104), most often by the recruitment and activation of various types of leukocytes (105). On the other hand, cys-LTs are smooth muscle contracting agents, particularly in the respiratory tract and microcirculation, and play pathogenic roles in acute and chronic asthma. This group of leukotrienes together with their receptors plays pivotal roles in several other disorders, such as allergic rhinitis, brain ischemia and in abdominal aortic aneurysm (104). In addition, cys-LTs are also associated with anaphylaxis, which involves mast cell activation and increased vascular permeability (106-109). Only very recently, cys-LTs have also been implicated in the development of celiac disease (110). Apart from the bioactive functions, biologically active leukotrienes can also be metabolized to inactive derivatives to facilitate elimination through enzymatic  $\omega$ -oxidation followed by  $\beta$ -oxidation (111).

## **2.2 BIOSYNTHESIS OF CYSTEINYL LEUKOTRIENES**

In this thesis, the focus will be on the biosynthesis of cys-LTs. Cysteinyl leukotriene biosynthesis originates from the breakdown of a membrane phospholipid into a polyunsaturated fatty acid named arachidonic acid (AA). The most common enzyme that catalyzes the hydrolysis of membrane phospholipids to liberate arachidonic acid is cytosolic phospholipase A<sub>2</sub>  $\alpha$  (cPLA<sub>2</sub> $\alpha$ ). 5-LO together with FLAP initiates the leukotriene biosynthesis from AA. FLAP does not have any known enzyme activity but is found to be important for

leukotriene biosynthesis. It is believed that FLAP can interact with 5-LO to activate and assist to deliver AA to 5-LO (112) in the nuclear envelope. 5-lipoxygenase (5-LO) uses AA as a substrate to convert it into 5-HpETE (Figure 6) by introducing molecular oxygen at C-5 position and LTA<sub>4</sub> is formed from 5-HpETE in the following dehydration step by the same enzyme, 5-LO (85). LTA<sub>4</sub> can either be used by leukotriene A<sub>4</sub> hydrolase (LTA<sub>4</sub>H) or by LTC<sub>4</sub>S. To synthesize the parent molecule, LTC<sub>4</sub>, for cys-LTs, LTC<sub>4</sub>S uses LTA<sub>4</sub> as a lipid substrate for conjugating it with reduced GSH to yield LTC<sub>4</sub>. The LTC<sub>4</sub> molecule synthesized within the cell is then transported over the plasma membrane by a probenecid-sensitive export carrier (113), which is identified as an ATP-dependent export pump for LTC<sub>4</sub> (114), a multi drug resistant protein (MRP1). LTC<sub>4</sub> can also be transported through another ATP-dependent export protein, MRP4, as seen in platelets, which is usually known as LTB<sub>4</sub> export pump in the presence of glutathione (115). LTC<sub>4</sub> then binds to its receptor on target cells or is further metabolized to LTD<sub>4</sub> and LTE<sub>4</sub> by the sequential cleavage of its tripeptide moiety by  $\gamma$ -glutamyl transpeptidase and dipeptidase, respectively. Cys-LTs exert their biological functions through their respective receptors. CysLT receptors are usually localized on the outer plasma membrane of target cells where they activate the G proteins to increase intracellular Ca<sup>2+</sup> or decrease intracellular cyclic AMP, which then leads to the activation of downstream kinases to trigger biological responses (100). CysLT1 and CysLT2 receptors are also present on eosinophil granule membranes (116). The important enzymes involved in this biosynthetic pathway are cPLA<sub>2</sub> $\alpha$ , 5-LO, FLAP and LTC<sub>4</sub>S. The soluble enzymes, cPLA<sub>2</sub> $\alpha$  and 5-LO, translocate to the nuclear envelope after cellular stimulation by a variety of stimuli. FLAP and LTC<sub>4</sub>S, are both localized in the nuclear membrane while LTC<sub>4</sub>S is only found in the outer nuclear envelope and ER membrane. It was reported previously that the leukotriene biosynthetic enzymes form multi-protein complexes upon cellular activation in the combinations of 5-LO, FLAP and LTC<sub>4</sub>S or 5-LO and FLAP in the outer (Figure 7) or inner nuclear membrane, respectively (117). All the LTC<sub>4</sub> biosynthetic enzymes are found in eosinophil lipid bodies, which indicates that cys-LT biosynthesis also can occur within the lipid bodies (118-121).

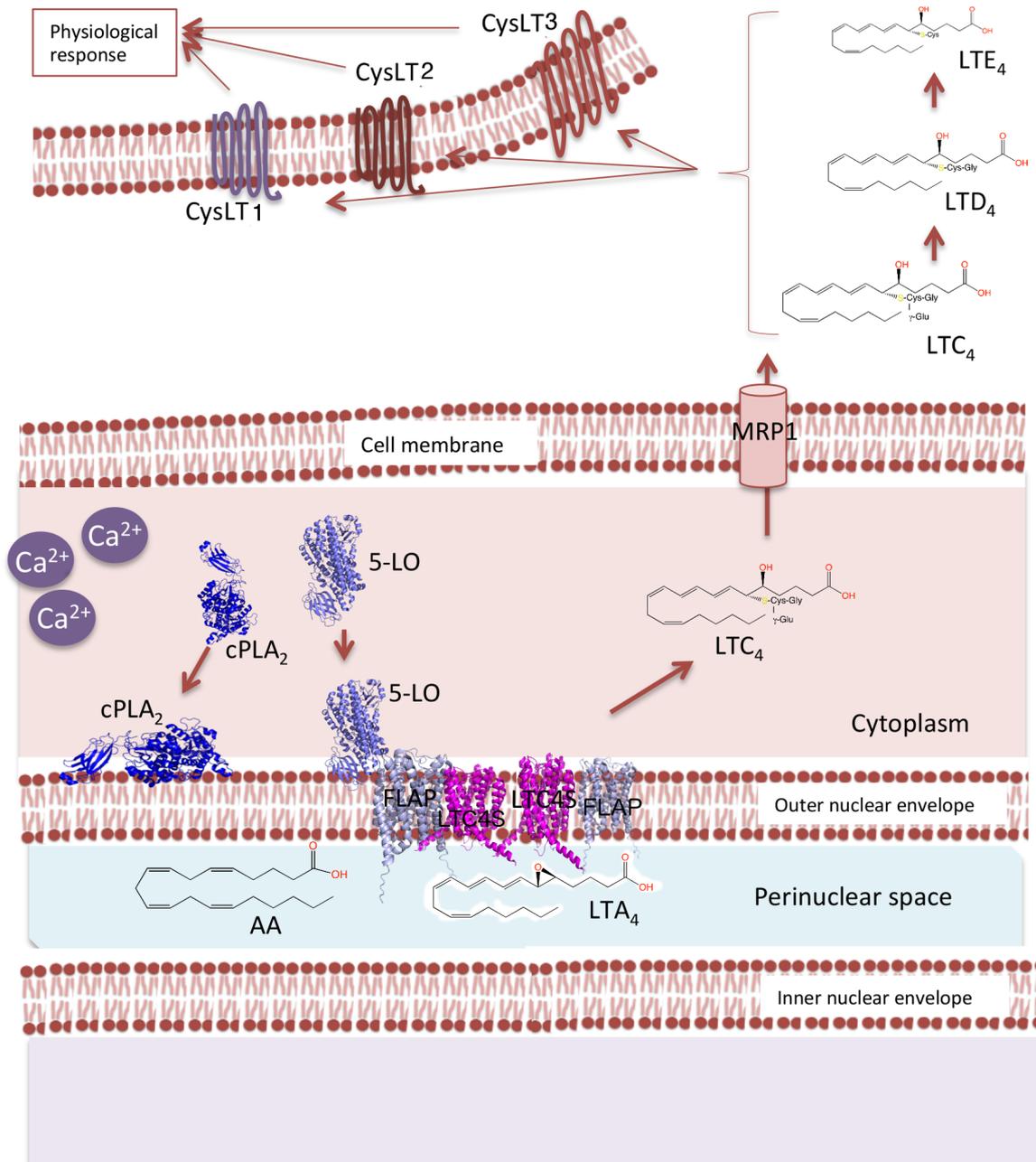


Figure 7: Cysteinyl leukotriene biosynthetic machinery. Upon cellular stimulation, the intracellular  $Ca^{2+}$  concentration increases which leads to the translocation of  $cPLA_2$  and 5-LO to the outer nuclear membrane. AA is released from the lipid bilayer by  $cPLA_2$  and presented to 5-LO with the assistance of FLAP.  $LTA_4$  is then produced enzymatically from AA by a two-step process catalyzed by 5-LO. An integral membrane protein  $LTC_4S$  (or  $MSGT2$ , depending on cell types) uses  $LTA_4$  as a substrate to conjugate it with a GSH molecule to produce  $LTC_4$ , which is then transported out from the cell through multidrug resistant protein 1 (MRP1).  $LTC_4$  is further metabolized to  $LTD_4$  and  $LTE_4$  and thus elicit their biological responses by binding to their respective receptors, such as CysLT1, CysLT2 and the recently discovered CysLT3.

The efficiency of this pathway has been shown to be regulated by factors, such as, the amount of free AA (122,123), the expression level of each of the proteins, modulation of enzyme activity by phosphorylation (124), and the presence of small molecules like ATP, nitric oxide (125), and reactive oxygen species to regulate 5-LO activity (100). Usually, non-leukocyte cells do not express 5-LO and FLAP sufficiently but express other leukotriene biosynthetic enzymes and in that case leukotriene biosynthesis occurs by transcellular biosynthesis where leukocyte-derived LTA<sub>4</sub> from donor cells (e.g., neutrophil) can be taken up by acceptor cells (e.g., endothelial cells) for further metabolism (126). However, LTA<sub>4</sub> is an unstable epoxide with a very short half-life ( $t_{1/2} \approx 10\text{s}$  at pH 7) (85) that needs to be transferred between the cells for transcellular biosynthesis. It has been shown that the stabilization of LTA<sub>4</sub> can be enhanced by a variety of molecules, such as albumin (127), cytosolic fatty acid-binding protein (128), and liposomes (129) to facilitate transcellular transport during cellular interaction. Some types of endothelial cells lack 5-LO and LTC<sub>4</sub>S but can produce LTC<sub>4</sub> by transcellular biosynthesis where MGST2 is found to be the one critical for LTC<sub>4</sub> production (75). Recently, another study reported a new MGST2 mediated LTC<sub>4</sub> biosynthetic machinery under ER stress (76).

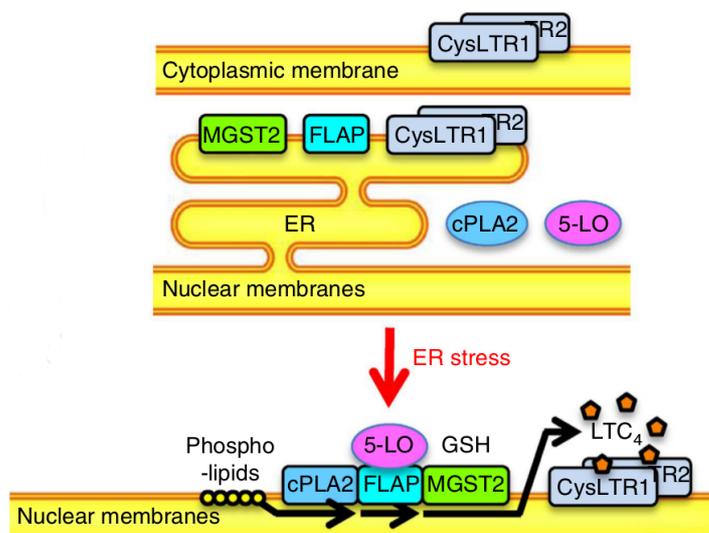


Figure 8: Newly proposed biosynthetic machinery for LTC<sub>4</sub> production by MGST2 under ER stress. The produced LTC<sub>4</sub> is suggested to bind to its internalized receptors. Figure adopted from (76).

## 2.3 ENZYMES IN LTC<sub>4</sub> BIOSYNTHETIC PATHWAY

### 2.3.1 cPLA<sub>2</sub> $\alpha$

The phospholipase A<sub>2</sub> (EC 3.1.1.4) enzyme superfamily consists of 15 different groups, separated based on their primary structure, localization and calcium dependencies. They are further classified into several major types including low-molecular-weight secreted (sPLA<sub>2</sub>), calcium dependent cytosolic (cPLA<sub>2</sub>), intracellular calcium independent (iPLA<sub>2</sub>), lipoprotein associated (Lp-PLA<sub>2</sub>), lysosomal PLA<sub>2</sub> and adipose-specific PLA (130-132). The enzymes in this superfamily specifically target the sn-2 acyl bond of phospholipids to release free fatty acids and lysophospholipids (132). Of these, group IVA cPLA<sub>2</sub> $\alpha$  is the major enzyme that is involved in the generation of free arachidonic acid for leukotriene biosynthesis (122,133). It can selectively hydrolyze the esterified arachidonic acid from the sn-2 position of membrane phospholipids to mediate agonist-induced liberation of arachidonic acid (134-136). In addition, it also displays lysophospholipase activity (132).

The cPLA<sub>2</sub> $\alpha$  is an 85 kDa soluble protein widely expressed in mammalian cells and tissues. Its expression can be modulated by certain cytokines, growth factors or corticosteroids (137). Ca<sup>2+</sup> ions play a crucial role for the activation and translocation of cPLA<sub>2</sub> $\alpha$  to the intracellular membranes in response to an elevation of intracellular Ca<sup>2+</sup> concentration. It can also be activated by ceramide-1-phosphate (C1P), phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and by phosphorylation at Ser-505 (138-140). There are more than one serine phosphorylation sites present in the catalytic domain and several studies have suggested that phosphorylation may regulate the catalytic activity and as well as the membrane binding (136).

The crystal structure of cPLA<sub>2</sub> $\alpha$  was determined at 2.5 Å resolution, which identified an N-terminal C2 domain and a C-terminal catalytic domain (141). The earlier solution structure determined for the N-terminal C2 domain revealed its  $\beta$ -sandwich structure with two Ca<sup>2+</sup> ions bound together with an extended binding site for a phosphocholine head group (142). The N-terminal C2 domain was also found to be homologous with the C2 domain of protein kinase C (PKC), which is thought to be important for its translocation. Therefore, it is believed that the N-terminal C2 domain is mainly a Ca<sup>2+</sup> dependent phospholipid-binding (CaLB) domain (143). It has also been shown experimentally that the N-terminal C2 domain is responsible for Ca<sup>2+</sup> binding and translocation to the membrane by using recombinant cPLA<sub>2</sub> $\alpha$  lacking the C2 domain or by mutating amino acids critical for Ca<sup>2+</sup> binding (138). The catalytic machinery consists of an unusual catalytic dyad formed by Ser-228 and Asp-549, located in a deep cleft at the center of a hydrophobic funnel of a C-terminal catalytic domain where a substrate can easily enter after binding to the membrane bilayer (141).

Another important residue, Arg-200, interacts with the phosphate group of the phospholipid substrate to stabilize substrate binding. The nucleophilic attack by Ser-228 at the sn-2 ester bond leads to the formation of an acyl-serine intermediate during the catalytic cycle where Asp-549 serves as a general base to increase the nucleophilicity of Ser-228 (111,132). An amphipathic loop has also been observed at the entrance of the active site, which acts as a flexible lid to allow substrate access by exposing the hydrophobic surface. It has also been found that anionic phospholipids can stimulate the enzyme activity by reducing the dependencies on  $\text{Ca}^{2+}$  ion requirements (144), which indicates its interfacial activation (85)

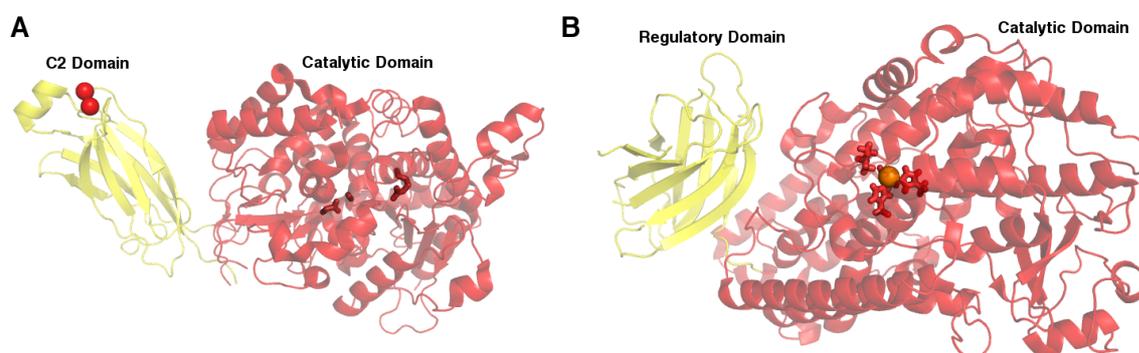


Figure 9: Structure of human cPLA<sub>2</sub>α (A) and human 5-LO (B). (A) The crystal structure of cPLA<sub>2</sub>α (PDB ID 1CJY) identified an N-terminal C2 domain (yellow) and a C-terminal catalytic domain (red). The C2 domain binds  $\text{Ca}^{2+}$  ions (shown in red spheres in the C2 domain) and is known as the CaLB domain. The catalytic dyad, Ser-228 and Asp-549, as well as Arg-200 are shown by stick representation at the active site of the catalytic domain. (B) The N-terminal regulatory/C2 like domain (yellow) and a C-terminal catalytic domain (red) are indicated in the crystal structure of stable 5-LO (PDB ID 3O8Y).  $\text{Ca}^{2+}$  ions bind to the C2 like domain with a stoichiometry of 2:1. The iron (orange sphere) is shown at the catalytic center located in the catalytic domain bound with iron coordinating residues (shown with stick): His-372, His-550, His-367 and Ile-673.

### 2.3.2 5-LO

5-lipoxygenase (5-LO) belongs to the lipoxygenase enzyme family, which are non-heme iron-containing dioxygenases. Most of the enzymes in that family catalyze the stereospecific incorporation of molecular oxygen into polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene moieties in the structure (145,146). Lipoxygenase enzymes are present in plants,

fungi and in animals. Several mammalian lipoxygenases are known for their critical roles in the biosynthesis of lipid hydroperoxides and signaling, such as 5-lipoxygenase (5-LO), 8-lipoxygenase (8-LO), 12-lipoxygenase (12-LO), and 15-lipoxygenase (15-LO) (146). The nomenclature of this enzyme family is based on their specificity for the substrates where the number of the carbon that is going to be dioxygenated is indicated and, if necessary, its stereoconfiguration is specified by either 'R' or 'S' (146).

The initial first two steps of the leukotriene biosynthesis from arachidonic acid are catalyzed by 5-LO through its two different catalytic functions. In the first step, molecular oxygen is inserted at C-5 by dioxygenase activity of 5-LO to form 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HpETE) and in the following step, an unstable epoxide intermediate 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, LTA<sub>4</sub>, is formed by the LTA<sub>4</sub> synthase activity (147).

5-LO is a soluble monomeric enzyme with a molecular mass of about 78 kDa (148), containing two domains, an N-terminal regulatory domain and a C-terminal catalytic domain (149,150). The N-terminal regulatory domain is also known as the C-2 like domain and displays similarity with lipases including cPLA<sub>2</sub>α (151). This domain is important for binding regulatory factors such as Ca<sup>2+</sup> (152), membrane phospholipids and coactosin like protein (CLP) (153) and plays a critical role in membrane interaction and translocation upon activation (154). The C-terminal catalytic domain contains the active site with a prosthetic iron (150,155). Iron coordinating residues are His-367, His-372, His-550 and Ile-673 (150,156-158), while the role of His-367 as an iron ligand is not crucial (156). The available crystal structures of mammalian lipoxygenases revealed that the positional specificity for the oxygenation of arachidonic acid may be determined by the depth and width of the substrate binding pocket (151).

The expression of 5-LO is limited to various types of leukocytes such as neutrophils, eosinophils, macrophages/monocytes, dendritic cells, mast cells, B-lymphocytes and certain foam cells (149). The enzyme expression is modulated under the influence of various cytokines and growth factors in different cell types (159-162).

One of the important regulatory factors for 5-LO activation is calcium as also seen for cPLA<sub>2</sub>α. The elevation of intracellular Ca<sup>2+</sup> ion concentration by various external stimuli triggers the translocation of both cPLA<sub>2</sub>α and 5-LO to the outer nuclear membrane, in close proximity to an integral membrane protein FLAP. This co-localization seems to be important for the efficient leukotriene biosynthesis to generate the precursor molecule LTA<sub>4</sub>. 5-LO

binds  $\text{Ca}^{2+}$  in a reversible manner with a stoichiometry of two  $\text{Ca}^{2+}$  per protein molecule (163). The  $\text{Ca}^{2+}$  activation of 5-LO requires the presence of phosphatidylcholine (PC) (164) or Coactosin Like Protein (CLP) (165). CLP, a 16 kDa protein, has been shown to bind 5-LO (166) and significantly increases the  $\text{LTA}_4$  synthase activity of 5-LO (167). It has been shown that  $\text{Ca}^{2+}$  increases the hydrophobicity of the C2 like domain of 5-LO by neutralizing negatively charged amino acids and mediating its interaction with the membrane through Trp-13, Trp-75 and Trp102 during translocation (168). 5-LO activity is also dependent on ATP, which seems to have a stabilizing effect by binding to the protein (85). Several studies have reported that 5-LO phosphorylation can be observed at three different sites, Ser-271 by MAPKAP kinase 2, Ser-663 by ERK2, and Ser-523 by PKA (124,169,170). Phosphorylation of 5-LO at Ser-663 and Ser-271 was shown to be important for subcellular localization and activation in certain cell types (170-172). In contrast, phosphorylation of 5-LO at Ser-523 inhibits the leukotriene production and nuclear import by shifting the subcellular distribution from the nucleus to the cytoplasm (124,173,174). Another study also reported that PKA mediated phosphorylation of 5-LO at Ser-523 favors the production of an anti-inflammatory mediator 15-epi-LXA<sub>4</sub> instead of leukotrienes (175). Subcellular localization of 5-LO is also an important determinant for the cellular leukotriene production where cytoplasmic 5-LO translocates to form multi-protein complex together with FLAP and LTC<sub>4</sub>S on the outer nuclear membrane to generate cys-LTs and nuclear 5-LO translocate to inner nuclear membrane in association with FLAP and favors LTB<sub>4</sub> biosynthesis (176,177). Cellular redox tone is found to be another important factor for the 5-LO product formation as the active ferric form ( $\text{Fe}^{3+}$ ) is maintained by lipid hydroperoxides (178). The 5-LO activity can also be regulated by a self-inactivation process as product inhibition by 5-HpETE (179) and  $\text{LTA}_4$  (180) through a suicide mechanism has been reported (111).

### **2.3.3 FLAP**

As mentioned in the previous chapter, FLAP is an integral membrane protein involved in leukotriene biosynthesis in association with 5-LO. FLAP is present only in cells that express 5-LO and possess leukotriene biosynthetic capacity (181), and is localized at the nuclear and ER membrane (182,183). It was first demonstrated as an important protein for the function of 5-LO in leukocytes. A potent leukotriene biosynthesis inhibitor, MK886, was found not to be specific for 5-LO in a cell free system but could prevent and revert the membrane translocation of 5-LO in intact cells (184). Later it was identified as a 18 kDa membrane protein, which was termed 'five-lipoxygenase activating protein' (FLAP). It was suggested

that it could bind to 5-LO at the membrane and play an essential role in transferring arachidonic acid to 5-LO (49). Another study has also shown that FLAP is essential for leukotriene biosynthesis (50). FLAP increases the ability of 5-LO to utilize arachidonic acid as a substrate and also enhances the LTA<sub>4</sub> synthase activity of 5-LO (185). The role of FLAP in the epoxide formation step by 5-LO could be due to the fact that FLAP protects 5-HpETE, produced in the dioxygenation step by 5-LO, from cellular peroxidases and facilitates its binding to the active site of 5-LO (111). It was suggested that each FLAP trimer could associate with one 5-LO monomer, through binding of the C-terminal catalytic domain of 5-LO with the cytosolic loop of FLAP, which sequentially transfers arachidonic acid to that 5-LO molecule (52).

#### **2.3.4 LTC<sub>4</sub>S AND MGST2**

The critical step in the biosynthesis of cys-LTs, the conjugation of LTA<sub>4</sub> with reduced GSH to produce LTC<sub>4</sub>, is mainly catalyzed by LTC<sub>4</sub>S (186). However, another membrane-bound glutathione transferase, MGST2, can also catalyze the formation of LTC<sub>4</sub> from LTA<sub>4</sub> as mentioned in the earlier chapter. The structural and functional properties of these integral membrane proteins have already been illustrated in the first chapter. LTC<sub>4</sub>S has been studied extensively for its crucial role in synthesizing cys-LTs in inflammatory cells of myeloid origin where all the proteins required for leukotriene biosynthesis are present (187). Platelets contain LTC<sub>4</sub>S but not 5-LO and are still able to produce LTC<sub>4</sub> through transcellular mechanism (188,189). On the other hand, MGST2 is generally abundant in non-haematopoietic cells. Recently, a novel MGST2-based LTC<sub>4</sub> signaling pathway has been identified, which is activated by ER stress and responsible for the stress-induced oxidative DNA damage (76).

#### **2.4 CYSTEINYL LEUKOTRIENE RECEPTORS**

Cysteinyl leukotrienes mediate their biological responses through binding to two major G-protein coupled receptors, CysLT1 and CysLT2, which differ in their sensitivity towards classical antagonists, such as montelukast, zafirlukast, pranlukast, pobilukast and MK571 (190). Two major types of Cys-LT receptors were initially classified solely on the basis of pharmacological studies but later they have been cloned, characterized and identified as two distinct receptors (191-195). Both of these receptors contain seven transmembrane helices and belong to the G protein-coupled receptor family. The CysLT1 receptor has higher affinity

towards LTD<sub>4</sub> ( $K_d \sim 1$  nM) while LTC<sub>4</sub> and LTE<sub>4</sub> bind with lower affinities (191). It expresses in various human tissues such as spleen, lung, placenta and small intestine and is found in several cell types like bronchial smooth muscle cells, monocytes/macrophages, mast cells, eosinophils, neutrophils and endothelial cells. In contrast, the CysLT2 receptor binds LTC<sub>4</sub> and LTD<sub>4</sub> with equal affinity ( $K_d \sim 10$  nM) while LTE<sub>4</sub> is a poor ligand (193). CysLT2 is found in various human tissues such as spleen, lung, heart, lymph node and brain. The tissue distribution and distinguishable ligand-binding properties of these two main CysLT receptors indicate that they have distinct roles in different cell types (196). CysLT1 and CysLT2 mediate signals by pertussis toxin (PTX)-sensitive and PTX-insensitive G proteins to exert their biological functions (196). They couple with G proteins of both class G<sub>q</sub> and G<sub>i</sub>, which leads to elevation of intracellular Ca<sup>2+</sup> and decreased intracellular cyclic AMP (cAMP). G protein  $\alpha$  subunits of G<sub>q</sub> class activate phospholipase C $\beta$  (PLC $\beta$ ) to initiate inositol phospholipid hydrolysis and Ca<sup>2+</sup> mobilization (197,198). On the other hand, G $\alpha_i$  subunit of G protein inhibits the enzyme adenylyl cyclase (AC) to block cAMP production by coupling with CysLT receptors upon ligand binding (199). Such modulations in the cellular environment activate downstream protein kinases to elicit respective cellular and tissue responses.

Several additional receptors have already been identified that show affinity for cys-LTs, such as CysLT3/CysLTE, P2Y<sub>12</sub> and GPR17 (85). CysLT3 and P2Y<sub>12</sub> have preference for LTE<sub>4</sub> to mediate their signals and could be poorly inhibited by CysLT1 selective antagonists (200-202). LTE<sub>4</sub> is a more stable and abundant cys-LT in cells, has powerful effects to induce mucosal eosinophilia and airway hyperresponsiveness in human asthma patients (202) but found to be a poor agonist for CysLT1 and CysLT2. It has been shown that the P2Y<sub>12</sub> receptor, an adenosine diphosphate reactive purinergic receptor, is essential for LTE<sub>4</sub>-induced pulmonary inflammation (202). Recently, identification of GPR99, a previously known oxoglutarate receptor (Oxgr1), as a potential CysLT3 receptor indicates its function as a LTE<sub>4</sub>-selective receptor to mediate vascular permeability (203). On the other hand, GPR17 was found to be a ligand-independent negative regulator of the CysLT1 receptor (204). However, It has been shown in another study that GPR17 does not have any cys-LT preference for binding and activation (205).

## 2.5 PHOSPHOREGULATION OF LTC4S

The regulation of enzyme activity by post-translational modification is a common phenomenon for many known enzymes. Reversible protein phosphorylation plays important roles in most signaling pathways to control cellular functions (206) and is also crucial for cys-LT biosynthesis by mediating activation and translocation of two key enzymes, cPLA<sub>2</sub> $\alpha$  and 5-LO, as described in the earlier sections. Another pivotal enzyme, LTC<sub>4</sub>S, in the cys-LT biosynthetic pathway, has shown to be regulated by reversible protein phosphorylation in different cell types (69,207,208). Initially, two putative protein kinase C phosphorylation sites were predicted based on the consensus site SerAlaArg when human LTC<sub>4</sub>S was cloned and sequenced from cDNA expression library in COS-7 cells (209). Several other studies have reported that treatment with phorbol-12-myristate-13-acetate (PMA), a known activator for protein kinase C, in the presence of calcium ionophore (A23187) specifically inhibits the production of LTC<sub>4</sub> in HL-60 cells and in human granulocytes (69,207,208). The PMA induced inhibition has been shown to be completely prevented in the presence of protein kinase inhibitor staurosporine. All these results suggest a protein kinase C dependent phosphoregulation of LTC<sub>4</sub>S in cells. It has also been demonstrated that phosphorylation of LTC<sub>4</sub>S occurs in a cell specific manner where it is observed in THP-1 cells but not in COS-7 or K-562 cells (70). A recent study suggested a late PGE<sub>2</sub>-mediated phosphoregulation of LTC<sub>4</sub>S, which might contribute to the resolution of inflammation (71). PGE<sub>2</sub> can activate downstream kinases by binding to PGE receptors and leads to the suppression of LTC<sub>4</sub> production via phosphorylation of LTC<sub>4</sub>S. A potential explanation for aspirin intolerant asthma has also been proposed based on such PGE<sub>2</sub>-mediated phosphorylation where aspirin can block PGE<sub>2</sub> production and subsequently activate LTC<sub>4</sub>S for cys-LT production. Using more selective kinase inhibitors, such as rapamycin, has identified another kinase pathway mediated by a ribosomal S6 kinase (p70S6k), which is also found to be involved in phosphoregulation of LTC<sub>4</sub>S. Thus, it was demonstrated that p70S6k can phosphorylate LTC<sub>4</sub>S both *in vivo* and *in vitro* (71).

## 2.6 LTC4S AND MGST2 AS POTENTIAL THERAPEUTIC TARGETS

Cys-LTs are potent pro-inflammatory mediators of bronchial asthma and allergic rhinitis (210) and influence many other biological responses. They are also associated with several other diseases, such as atopic dermatitis (211), allergic conjunctivitis (212), celiac disease (110) and anaphylaxis (108). Moreover, involvement of cys-LTs and their receptors in cardiovascular disease and cancer have been reported. Thus, it was demonstrated that cys-

LTs participate in the development of atherosclerotic lesions and may increase the risk of cancer by chronic inflammation (100). At present, the available anti-leukotriene drugs in this pathway are mainly targeting 5-LO (Zileuton), FLAP and the cys-LT receptors. CysLT1 antagonists, referred to as the 'lukast' class of drugs (Montelukast, Zafirlukast and Pranlukast) are currently used for treatment of asthma and allergic rhinitis. It has been reported that 40% of asthma patients do not respond to lukast agents (85). The notable expression of the CysLT<sub>2</sub> receptor in the brain, adrenals, heart and vascular endothelium indicates additional unknown functions of cys-LTs, which could affect the potency of these drugs. The limitations of these therapeutic agents towards certain group of patients could also be due to the presence of several other CysLT receptors, such as CysLT3 and gpr17, and their complex signaling mechanisms. Therefore, LTC4S has become a promising therapeutic target, as its antagonists would be more specific to block the production of cys-LTs by avoiding such complexities. Moreover, identification of MGST2-based LTC<sub>4</sub> biosynthetic machinery and observed LTC<sub>4</sub> production in cells devoid of LTC4S may expand the possibilities of therapeutic application for new agents towards MGST2.

### 3 EXPERIMENTAL BACKGROUND

#### 3.1 ENZYME KINETICS

Enzyme kinetics deals with the rates and equilibrium constants of enzyme-catalyzed reactions under different conditions to understand physiological functions and specificity of an enzyme. The study of enzyme kinetics allows us to deduce the mechanism of the catalytic action of an enzyme and its affinities towards different substrates and inhibitors. Some basic principles of enzyme kinetics are briefly discussed in this section.

##### 3.1.1 Steady-state kinetics

Generally, the enzyme catalyzed reaction can be described by a simplified model as shown below where enzyme binds substrate to form an enzyme-substrate complex (ES) in the first step, and in the following step, the enzyme-substrate complex undergoes a reaction and dissociates to yield product and free enzyme.



Initially, Michaelis-Menten proposed a simple model for enzyme-catalyzed reactions by measuring initial rates of the reaction at different substrate concentrations at controlled pH, which was based on the assumption that enzyme and substrate associate reversibly to form ES complex assumed to be a rapid equilibrium represented by dissociation constant  $K_s$ . Later, Briggs and Haldane introduced the ‘steady-state assumption’ to refine the Michaelis-Menten equation to the present form (213). According to their argument the ES complex forms and dissociates rapidly with the same rate by which it reached to a steady-state. Thus, the concentration of ES complex remains approximately constant over time during the steady state. Such theoretical state can be achieved by using great excess of substrate concentration compared to enzyme concentration where the free substrate concentration will remain almost equal to the amount added. In addition, based on the initial velocity assumption the total enzyme concentration can be expressed as a sum of two species, free enzyme and the enzyme-substrate complex. By considering all these theoretical conditions the following Michaelis-Menten equation is now deduced for the enzyme-catalyzed reactions during steady-state phase.

$$v = \frac{k_{\text{cat}} [E]_0 [S]}{K_m + [S]}$$

The steady-state kinetic measurements are limited by describing the kinetic and mechanistic information related to a minimal mechanism where no direct information could be obtained for the formation/disappearance of intermediates within the reaction pathway. The important information in this method is provided in the form of kinetic parameters,  $k_{\text{cat}}$ ,  $K_m$  and  $k_{\text{cat}}/K_m$ .

### 3.1.2 Pre-steady state kinetics

The kinetic parameters obtained from the steady-state kinetic analysis are complex functions of all the reaction rates involved in the catalytic pathway (214). The individual rates cannot be resolved independently by steady-state analysis. In contrast, the pre-steady-state kinetics allows investigating the fast reactions on a short time scale through which limitations arising from steady-state kinetics can be overcome. This method is usually employed to analyze the individual steps in the reaction pathway where stoichiometric amount of enzyme and substrate are rapidly mixed to enable the direct detection of each intermediate and product formed in various steps during a catalytic cycle (215).

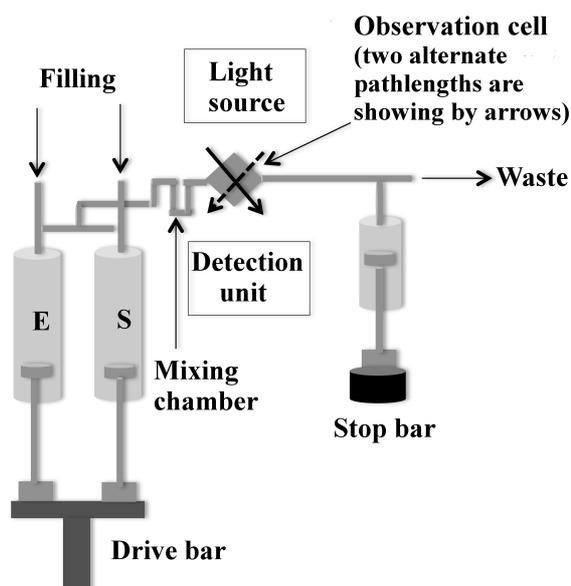


Figure 10: Schematic diagram of a stopped-flow apparatus.

There are several techniques that can be used to study the fast reactions ranging from around nanosecond to millisecond timescale. Among them, stopped-flow and rapid-quench-flow are the two most common techniques used for studying fast reactions on a millisecond time scale. In the stopped-flow method, after rapid mixing of enzyme and substrate, one can measure the formation of short-lived species by following the changes in optical signals (typically absorbance, fluorescence and light scattering) as a function of time. The analysis of observed rates and amplitudes of such detectable short-lived species would allow defining the reaction pathway after substrate binding to product release (215).

### 3.1.3 Inhibition kinetics

Many Pharmaceutical compounds and drug molecules are known as inhibitors as they can negatively alter the enzyme activity by binding to the target proteins. Kinetic analysis of enzymes in the presence of such inhibitors assist to reveal the information about their potency and mode of interaction with enzyme molecules (216). The reversible inhibitors are classified into several types based on their effects on the kinetic parameters,  $K_m$  and  $V$ . A competitive inhibitor increases  $K_m$  without affecting  $V$  while completely opposite behavior is observed with a non-competitive inhibitor. On the other hand, both  $K_m$  and  $V$  are affected in the presence of uncompetitive inhibitor as it only binds to the ES complex. A competitive inhibitor binds to the enzyme active site and compete with substrates. In contrast, inhibitors can bind at different sites (both free E and ES complex) of the enzyme with equal affinity during non-competitive inhibition. Another class is termed as mixed inhibition, where inhibitors bind to free E and ES complex with different affinity and thus alter both apparent  $K_m$  and apparent  $V$ . The inhibition constant  $K_i$ , which is independent of substrate concentration, is basically a dissociation constant of EI or ESI complex and can be represented as shown below.

$$K_{ia} = \frac{[E][I]}{[EI]}, \quad K_{ib} = \frac{[ES][I]}{[ESI]}$$

Besides, the term  $IC_{50}$  is most frequently used to define the apparent inhibitor potency in pharmacological investigations. It represents the inhibitor concentration required for the inhibition of enzyme activity by one half at a fixed substrate concentration. The  $IC_{50}$  value is dependent on substrate concentration and its  $K_m$  except for the non-competitive inhibitor (216,217).

### **3.1.4 Global kinetic simulation**

Global kinetic simulation allows fitting multiple kinetic data sets simultaneously to a catalytic model based on numerical integration of the rate equations (218). The data fitting process includes all the information obtained from the experiments, such as rates and amplitudes, without any simplified assumption, which is advantageous over the conventional data fitting process. In this process, the experimental data (e.g. the optical signals as a function of time at different substrate concentrations) are directly fit to the model by including appropriate scaling and output factors to relate the observable signals to the absolute amount of reactants (219). Consequently, It enables the determination of all the rates and equilibrium constants globally to deduce a complete catalytic mechanism for an enzyme catalyzed reaction.

## **3.2 EQUILIBRIUM DIALYSIS**

Equilibrium dialysis enables the study of binding of low molecular weight molecules and ions to the protein under equilibrium conditions. The stoichiometry of ligand binding and dissociation constant,  $K_d$ , to estimate the affinity of ligands for the proteins, can be obtained directly from this experimental technique. In this method, protein and ligand molecules are usually placed into two identical chambers separated by a semipermeable membrane. The membrane only allows the low molecular weight ligands to diffuse through it from the high concentration chamber to the low concentration chamber. At the time of equilibrium, the concentration of free ligand becomes equal in both chambers. The amount of bound ligand to the protein can then be calculated by measuring the concentration of ligands in each chamber at equilibrium.

## **3.3 ISOTHERMAL TITRATION CALORIMETRY**

Isothermal titration calorimetry (ITC) is a powerful and sensitive technique to study biomolecular interactions and is frequently used in drug discovery to identify the lead compounds. The basic principle of this method is based on the measurement of heat released or absorbed in response to the biomolecular interactions. A typical ITC instrument consists of reference cell (usually contains water), sample cell (often contains protein) and a syringe for the addition of titrants. The instrument monitors heat changes after each addition of titrant to the sample cell by measuring the applied power to the cell heaters to maintain the constant temperature between two cells (220). This method is effective as it allows determining the binding and thermodynamic parameters in a single in-solution experiment in which no

modifications of biomolecules are needed. The important parameters that can be obtained by ITC experiment are binding affinity ( $K_d$ ), stoichiometry (N), enthalpy ( $\Delta H$ ), and entropy ( $\Delta S$ ). ITC has also been found to be an important biophysical technique to study the thermodynamics of cooperativity in biological interactions (221) and enzyme kinetics (222).

### **3.4 DIFFERENTIAL SCANNING CALORIMETRY**

Differential scanning calorimetry (DSC) is another powerful calorimetric technique usually employed to study the biomolecular stability. This technique can also be applied to analyze the biomolecular interactions (223) and oligomeric state of the functional unit of a protein (Paper III). It measures the excess heat capacity ( $C_p$ ) of a solution that contains molecules of interest as a function of temperature (T). A sharp endothermic peak can describe the thermally induced transitions of a biological macromolecule between the folded and unfolded state and the melting temperature ( $T_m$ ) can be obtained from the temperature at maximum  $C_p$ .  $T_m$  represents the temperature at which half of the molecules are in native state or denatured. The calorimetric enthalpy ( $\Delta H^\circ_m$ ) of such transition and heat capacity change ( $\Delta C_p$ ) can be determined directly by integration of the  $C_p$  versus T thermogram and from the shift in the base line, respectively. The van't Hoff enthalpy ( $\Delta H^\circ_{vH}$ ) of transition can be estimated from the DSC thermogram by using van't Hoff equation based on an assumed model. In addition, important information related to transition process and oligomeric state of a macromolecule can be obtained by comparing the  $\Delta H^\circ_m$  with  $\Delta H^\circ_{vH}$  (223).

### **3.5 BLUE NATIVE PAGE**

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a useful method to isolate protein complexes from various sources like biological membranes, cell and tissue homogenates. It is also used for other purposes, such as for the determination of molecular mass and oligomeric state of a native protein and to study physiologically occurring protein-protein interactions (224). In this method, the electrophoretic mobility of a protein in the polyacrylamide gel is mainly influenced by the negative charge of bound Coomassie blue dye and thus known as the charge shift method. Coomassie blue binds to the hydrophobic surface of the protein to induce charge shift and to protect membrane proteins from aggregation and denaturation (225).

### **3.6 MASS SPECTROMETRY**

Mass spectrometry is a sensitive analytical technique widely used in various fields including proteomics. It allows detection, identification and quantification of molecules based on their mass/charge ( $m/z$ ) ratios. Using mass spectrometry (MS)-based techniques for the analysis of phosphopeptide detection and sequencing of phosphorylated protein samples are not very straightforward for several reasons, such as poor signals from phosphopeptides due to the abundance of nonphosphorylated species, weak ionization of phosphopeptide, presence of isobaric peptides etc. (226). However, using different phosphopeptide enrichment strategies and combination of liquid chromatographic steps before MS/MS analysis along with proper fragmentation processes can overcome the difficulties with assigning the phosphorylation sites. The detail descriptions of the process are described in paper IV.

### **3.7 MOLECULAR DYNAMICS SIMULATION**

Molecular dynamics simulations are now frequently used to aid in understanding dynamic processes of membrane proteins that are difficult to observe experimentally. The recent advancement in this field enables the routine running of simulations of membrane proteins embedded within lipid bilayers. In a molecular dynamic simulation, Newtonian motion of molecules is modeled with a force field for a period of time (ns- $\mu$ s). The known structure of a protein molecule is commonly used as an initial structure and prepared for the simulation after energy minimization. Preparation of protein and lipid bilayer, insertion of protein in the lipid bilayer and establishing a stable system are prerequisites before final run.

### **3.8 PROTEIN CRYSTALLOGRAPHY**

Structural information of a protein molecule is very essential to understand its physiological function and molecular mechanism. Protein crystallography is a technique by which the crystal structure of a protein molecule can be determined through a series of sophisticated iterative processes. The description of all the relevant steps in the determination of crystal structures of proteins, are beyond the scope of this section but an overview of this technique is presented through a flow chart in the next page.

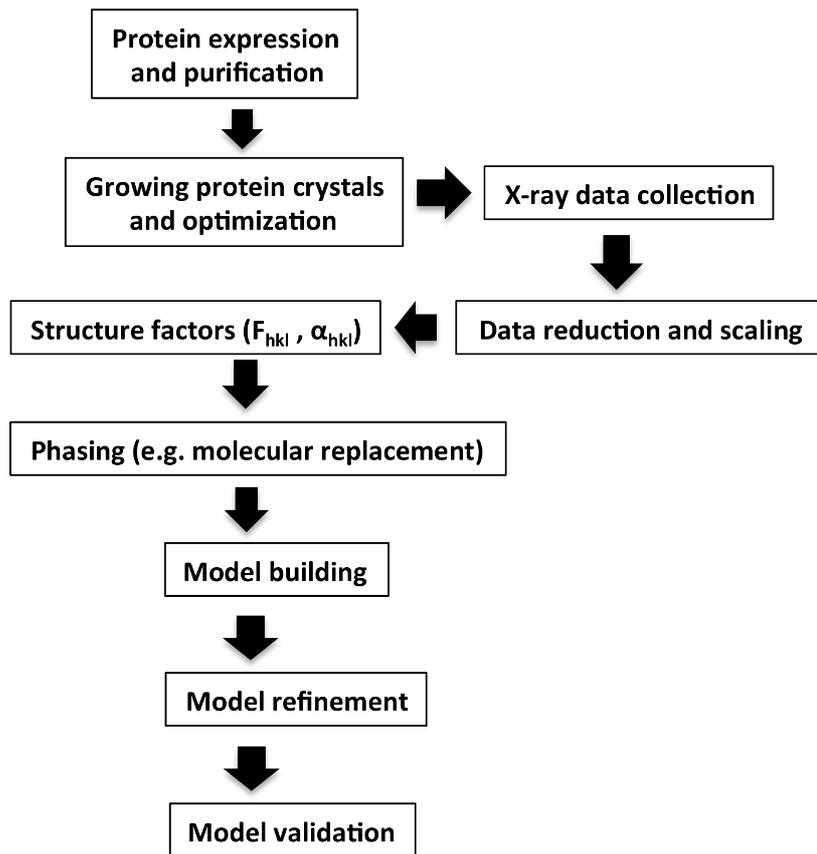


Figure 11: The step-by-step processes involved during the structure determination of a protein.

## 4 AIM OF THE STUDY

As mentioned earlier, LTC4S and MGST2 have become attractive drug targets for their crucial role in cys-LT biosynthesis. Both of these proteins are members of the MAPEG superfamily. MAPEG family proteins are involved in various metabolic processes and play important roles in the biosynthesis of lipid mediators. The overall aim of the present thesis is to understand the regulatory and molecular mechanism of enzymes involved in cys-LT biosynthesis, mainly LTC4S and MGST2, to facilitate effective development of drugs against inflammatory diseases including asthma and allergic disorders. The knowledge from these two proteins could also be used to understand the mechanism of other MAPEG members and to study the functional relevance among them. The individual aims for each paper are as follows.

**Paper I:** To study the mechanistic details of glutathione binding and activation of LTC4S.

**Paper II:** To explore substrate specificity and investigate the catalytic mechanism of MGST2.

**Paper III:** To get an overview of the functional unit and establish the detailed mechanism of 1/3 of the sites reactivity of MGST2.

**Paper IV:** To investigate the molecular mechanism behind phosphoregulation of LTC4S.

## 5 RESULTS AND DISCUSSION

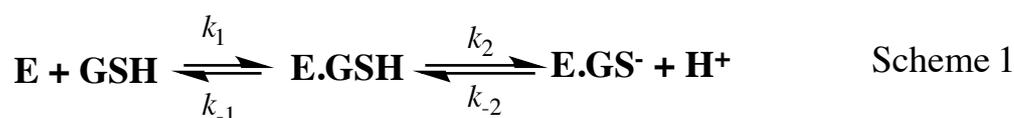
### 5.1 MECHANISM OF GSH BINDING AND ITS ACTIVATION (PAPER I & II)

The basic requirement of the catalytic mechanism of all GSTs is the binding of GSH and its activation to form a thiolate anion ( $\text{GS}^-$ ) at physiological pH.  $\text{GS}^-$  acts as a nucleophile to attack at the electron deficient carbon of the electrophilic substrate. This property has been investigated for both LTC4S and MGST2. The spectroscopic evidence for the formation of  $\text{GS}^-$  was demonstrated previously by UV difference spectroscopy for LTC4S (66) and it has now been shown for MGST2. A UV difference spectrum ranging from 210 to 300 nm was recorded after mixing MGST2 with GSH at pH 7.2 and the  $\text{GS}^-$  was detected by the increase in the absorbance at 239 nm. The absence of the increase in the absorbance at 239 nm in the presence of an inhibitor, glutathione sulphonic acid ( $\text{GSO}_3^-$ ), confirmed that the change in the absorbance is due to the presence of  $\text{GS}^-$ . The  $\text{GSO}_3^-$  has been shown to be a competitive inhibitor for both LTC4S and MGST2 and occupy the same site as GSH with an identical bent conformation, which is confirmed by the crystal structure of LTC4S in complex with  $\text{GSO}_3^-$ . Both of these proteins have the ability to lower the  $\text{pK}_a$  of enzyme bound GSH to form  $\text{GS}^-$  by deprotonation. The  $\text{GS}^-$  formation was observed at 239 nm, both for LTC4S (66), and MGST2 (Paper II), by UV difference spectroscopy. Generally, the  $\text{pK}_a$  of GSH in solution is around 9 (227). Pre-steady-state stopped-flow kinetics is used to determine the  $\text{pK}_a$  of enzyme bound GSH where the amount of  $\text{GS}^-$  formed was calculated from the amplitude at 239 nm at different pH after rapid mixing of enzyme with GSH. The  $\text{pK}_a$  of enzyme bound GSH was found to be 5.9 and 6.3 for LTC4S and MGST2, respectively. The observed  $\text{pK}_a$  values are similar to those of unactivated (5.7) and activated (6.3) MGST1 (35) and are within the range ( $\text{pK}_a = 6-7$ ) previously reported for other GSTs (227-229). The mechanism of these enzymes is therefore also likely to be facilitated by the lowering of enzyme bound GSH thiol  $\text{pK}_a$  to that of physiological pH. The key catalytic residue involved in the stabilization of  $\text{GS}^-$  was identified as Arg-104 for LTC4S (66). The peak at 239 nm for  $\text{GS}^-$  was absent in the UV difference spectrum for the mutants R104A and R104S and the role of Arg-104 was further confirmed by kinetic analysis together with the pH dependency of the specific activity of WT and mutant enzymes. At higher pH, the inactive mutant should retain some activity as the enzyme bound GSH would then be able to form  $\text{GS}^-$  spontaneously. Indeed, this was observed for the R104A and R104S mutants. The structure of LTC4S, together with the kinetic data, indicates that  $\text{GS}^-$  formation in LTC4S is solely aided by a positively charged Arg-104 through electrostatic stabilization. Arg-104 is conserved within

the MAPEG super family with the exception of FLAP. This conserved arginine (Arg-126) has also been identified as an important residue for the stabilization of GS<sup>-</sup> in mPGES1 (47). Therefore, the observed role for this particular residue in LTC4S suggests its common functional role as a GS<sup>-</sup> stabilizing residue within other MAPEG members.

The comparison between crystal structures of the LTC4S complex alternatively with GSH and GSO<sub>3</sub><sup>-</sup> demonstrated that GSH binding within the active site is slightly different than that of GSO<sub>3</sub><sup>-</sup> binding in terms of interaction with Arg-104. The DDM molecule (mimic of lipid substrate LTA<sub>4</sub>) is absent in the structure with GSO<sub>3</sub><sup>-</sup>, which could be due to the fact that the bulky sulfate of GSO<sub>3</sub><sup>-</sup> hinders the access of DDM to the active site. This could be an indication that the proper positioning of GSH and its binding is essential to attain productive binding of LTA<sub>4</sub> with high selectivity for efficient catalysis of LTC4S.

Binding of GSH and thiolate anion activation can be described by a two-step mechanism (Scheme 1). The first step is the rapid equilibrium formation of the initial E.GSH complex, followed by the E.GS<sup>-</sup> complex formation step.



The individual rate constants for the GSH binding and GS<sup>-</sup> formation were obtained by the pre-steady-state kinetics where enzyme was rapidly mixed with GSH to follow the formation of GS<sup>-</sup> at 239 nm using a stopped-flow spectrophotometer. The  $K_d^{\text{GSH}}$  ( $k_{-1}/k_1$ ) obtained for LTC4S and MGST2 were  $3.0 \pm 0.6$  mM and  $4.3 \pm 0.6$  mM, respectively, which are comparatively lower than that observed for MGST1 ( $11.7 \pm 3.1$ ) (37). Rapid formation of GS<sup>-</sup> was observed for LTC4S ( $k_2 = 200$  s<sup>-1</sup>) relative to MGST2 ( $k_2 = 41.1$  s<sup>-1</sup>) and was not rate limiting for the overall catalysis, whereas this step was found to be rate limiting for MGST1 catalyzed reactions towards reactive secondary substrates (36).

The rate of GSH release ( $k_{-2}$ ) from the enzyme-thiolate complex was determined independently by a stopped-flow rapid dilution experiment of premixed enzyme in equilibrium with a nonsaturating amount of GSH, which can be followed by the loss of

absorbance at 239 nm. The  $k_{-2}$  obtained for both LTC4S ( $1.3 \text{ s}^{-1}$ ) and MGST2 ( $2 \text{ s}^{-1}$ ) were faster compared to that of the  $k_{-2}$  observed for MGST1 ( $0.016 \text{ s}^{-1}$ ).

The binding affinity for  $\text{GS}^-$ ,  $K_d^{\text{GS}^-}$ , was determined experimentally and compared with the theoretical value to validate the model used for GSH binding and activation. The theoretical  $K_d^{\text{GS}^-}$  was calculated from the equation 1 by using the microscopic rate constants ( $k_2$  and  $k_{-2}$ ) and the equilibrium constant ( $K_d^{\text{GSH}}$ ). The theoretical and the experimental values for both LTC4S and MGST2 were in good agreement and suggested that our model for GSH binding and activation was correct.

$$K_d^{\text{GS}^-} = \frac{K_d^{\text{GSH}}}{1 + \frac{k_2}{k_{-2}}} \quad (1)$$

The proton released from the GSH during  $\text{GS}^-$  formation can either be abstracted by a residue within the protein or released to the solvent. The structure of LTC4S identifies Arg-104 as the only basic residue located in close proximity to the cysteinyl thiol of GSH and its high  $\text{p}K_a$  ( $\sim 12$ ) suggests that the proton is released to the solvent upon  $\text{GS}^-$  formation. The observed rate ( $k_{\text{obs}} = 18 \text{ s}^{-1}$ ) of proton release was measured and found to be similar with the  $k_{\text{obs}}$  ( $14 \text{ s}^{-1}$ ) of  $\text{GS}^-$  formation at the same enzyme and GSH concentration for LTC4S. The amount of proton released was quantified by potentiometric titration and showed that one  $\text{GS}^-$  was formed per LTC4S monomer. The similar rate indicated that the released proton originated from GSH and that it was probably not a rate-limiting factor for LTC4S catalysis. It could be possible that this proton contributes to the oxyanion protonation at C5 of  $\text{LTA}_4$  by proton shuffling during the conjugation reaction (65). Conversely, the observed rate ( $k_{\text{obs}} = 9 \text{ s}^{-1}$ ) of proton release was slow compared to the  $k_{\text{obs}}$  ( $18 \text{ s}^{-1}$ ) of  $\text{GS}^-$  formation at the same enzyme and GSH concentration for MGST2, which indicates that proton release could be a rate-limiting factor for enzyme catalyzed reactions having a rapid chemical step. Protonation/deprotonation is also a very rapid process and thus, the observed slow rate for proton release could be due to the rate-limiting conformational transitions associated with GSH activation.

The amplitude analysis of all the signals at 239 nm obtained from several different experimental setups, such as UV difference spectra, stopped-flow thiolate anion formation at 239 nm, as well as the amplitude of initial burst at 340 nm from active site titrations provide valuable stoichiometric information about  $\text{GS}^-$  formation per trimeric enzyme. All three

monomers of LTC4S are competent to activate GSH into GS<sup>-</sup> while only one GS<sup>-</sup> is formed per trimeric MGST2. The observed GS<sup>-</sup> / enzyme subunit stoichiometry of 1/3 has also been previously observed for two other MAPEG members, MGST1 and MPGES1. The observed one-third-of-the-sites reactivity of MGST2 is further characterized in Paper III.

## 5.2 SUBSTRATE SPECIFICITY AND CATALYTIC MECHANISM OF MGST2 (PAPER II)

MGST2 is homologous to LTC4S and both of these proteins catalyze the biosynthesis of LTC<sub>4</sub>. LTC4S has been well characterized with respect to its function and specificity towards LTA<sub>4</sub>. It has evolved mainly as the major LTC<sub>4</sub> biosynthetic enzyme while the central physiological role for MGST2 is yet unclear. The substrate specificity and catalytic properties of purified human MGST2 have been characterized in this study for the first time.

In all previous studies, the LTC4S activity of MGST2 was only analyzed in membrane preparations of the enzyme (72,78). In this study, the LTC<sub>4</sub> production was analyzed by purified MGST2 to compare its efficiency with LTC4S. The results showed that the specific activity of MGST2 (1.2 μmol min<sup>-1</sup> mg<sup>-1</sup>) towards LTA<sub>4</sub> is only 3.4% relative to that of LTC4S (35 μmol min<sup>-1</sup> mg<sup>-1</sup>). However, the comparison of LTC4S activity of MGST2 with other GSTs, such as cytosolic GSTs (2-10 nmol min<sup>-1</sup> mg<sup>-1</sup>) (230,231) and MGST1 (0.2 nmol min<sup>-1</sup> mg<sup>-1</sup>) (78), suggests that MGST2 could be involved in the biosynthesis of LTC<sub>4</sub> at physiological conditions and is now confirmed in a recent study, showing MGST2 mediated LTC<sub>4</sub> formation under ER stress in non-haematopoietic cells (76). The second order rate constant,  $k_{cat}/K_m^{LTA_4}$ , displayed by MGST2 and LTC4S were  $1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  and  $8.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. Hence, MGST2 is about 48 times less efficient than LTC4S in productive substrate binding towards LTA<sub>4</sub>. However, the  $K_m^{LTA_4}$  was not significantly different for MGST2 (40 μM) compared to that of LTC4S (30 μM).

MGST2 also possesses GSH-dependent hydroperoxidase activity, which is investigated using lipid hydroperoxides, such as 5-HpETE, 15-HpETE, 13-HpODE and 13-HpOTrE, as substrates. The catalytic efficiency obtained with these lipid hydroperoxides by MGST2 ranged from  $0.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  to  $1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  and the  $K_m$  values observed were within the range of 5-20 μM, as seen for other GSTs such as hGSTA1-1 (232). These results suggest that MGST2 could also be involved in the reduction of lipid hydroperoxides to maintain cellular redox tone, which is an important factor for regulating 5-LO and cyclooxygenase activity in arachidonic acid metabolism (178,233).

MGST2 catalyzes the conjugation of GSH with 1-Chloro-2,4-dinitrobenzene (CDNB) ( $37.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ) and with 4-Hydroxy-2-nonenal (4-HNE) ( $0.9 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ). CDNB is a universal substrate commonly used for characterizing GSTs, while 4-HNE is a product from lipid peroxidation and mediator of oxidative stress (234). The specific activity obtained for 4-HNE by MGST2 was comparatively lower than the activity reported for some soluble GSTs, however similar activity was displayed by MGST1 (27). In contrast, hardly any detectable activity could be obtained when 4-HNE and CDNB were provided to LTC4S as substrates. Thus, MGST2 differs from LTC4S largely by its ability to conjugate GSH with toxic xenobiotic, and lipid peroxidation products, to form neutralized adducts for excretion. MGST1 and MGST2 are abundant in the liver and hence, both of these proteins could have shared roles as xenobiotic metabolizing enzymes.

A series of CDNB like substrates have been used to study the effect of chemical reactivity of second substrate to the catalytic efficiency of MGST2. The CDNB like substrates, such as 4-chloro-3-nitrobenzaldehyde (CNBAL), 4-chloro-3-nitrobenzacetophenone (CNAP) and 4-chloro-3-nitrobenzamide (CNBAM), are electron deficient aryl substrates, only differing by the *para*-substituent chemical groups relative to the common leaving group (Cl). CDNB is the most reactive because of the presence of electron-withdrawing *para*-nitro ( $\text{NO}_2$ ) group. The catalytic properties,  $k_{\text{cat}}^{\text{E}}$  and  $k_{\text{cat}}/K_{\text{m}}^{\text{E}}$  ( $k_{\text{cat}}^{\text{CDNB}} = 14.3 \text{ s}^{-1}$ ,  $k_{\text{cat}}/K_{\text{m}}^{\text{CDNB}} = 7.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ;  $k_{\text{cat}}^{\text{CNBAL}} = 1.6 \text{ s}^{-1}$ ,  $k_{\text{cat}}/K_{\text{m}}^{\text{CNBAL}} = 2.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ;  $k_{\text{cat}}^{\text{CNAP}} = 0.3 \text{ s}^{-1}$ ,  $k_{\text{cat}}/K_{\text{m}}^{\text{CNAP}} = 3.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{\text{cat}}^{\text{CNBAM}} = 0.17 \text{ s}^{-1}$ ,  $k_{\text{cat}}/K_{\text{m}}^{\text{CNBAM}} = 0.9 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ ), were used in the Hammett plot to check whether the chemical step is rate limiting for the MGST2 catalysis with these substrates. The logarithm of rate constants were plotted against the Hammett substituent constant ( $\sigma^-$ ) and resulted in a linear relationship. The analysis of Hammett  $\rho$  values (2.8 for non-enzymatic conjugation rate, 2.9 for  $k_{\text{cat}}^{\text{E}}$  and 4.3 for  $k_{\text{cat}}/K_{\text{m}}^{\text{E}}$ ) reflect the influence of chemical reactivity on the rate constants and thus, suggest that the chemical step is contributing to turnover at both saturating and non-saturating concentrations of the electrophilic substrates.

GSH binding and thiolate anion formation have been kinetically characterized for both LTC4S and MGST2 by pre-steady-state kinetics whereas the chemical conjugation step of LTC4S could not be studied in detail because of its unstable substrate,  $\text{LTA}_4$ . On the other hand, CDNB was used as a substrate to study the chemical conjugation step of MGST2 by pre-steady-state kinetics. The enzymatic chemical conjugation step of MGST2 can also be described by a simple two-step mechanism with a rapid equilibrium (E.GS.CDNB complex forming) step followed by the chemical step (Scheme 2) as described for the related enzyme

MGST1 (36). Rapid mixing of E.GS<sup>-</sup> complex with CDNB in a stopped-flow instrument allowed us to follow the signals of initial burst of product formation followed by a steady-state rate at 340 nm. The initial burst of product formation suggests a rate-limiting factor after product formation, which could be due to the product release or recharging of thiolate. The product release (~118 s<sup>-1</sup>) was measured directly by following the fluorescence change during the rapid dilution experiment using stopped-flow with enzyme-product complex (E.GSDNB) and found not to be rate limiting for MGST2 catalysis. The initial burst is more pronounced at low GSH concentration and this could be due to the fact that the recharging of thiolate becomes the rate-limiting factor at that point. In contrast, initial burst of product formation was absent with CNAP indicating chemistry being the rate-limiting step for MGST2 catalysis towards less reactive substrates.



$$k_{\text{obs}} = \frac{k_4 [\text{CDNB}]}{K_d^{\text{CDNB}} + [\text{CDNB}]} \quad (2)$$

$$k_{\text{obs}} = \frac{k_4 [\text{CDNB}]}{K_d^{\text{CDNB}}} \quad (\text{Where } K_d^{\text{CDNB}} \gg [\text{CDNB}]) \quad (3)$$

The rates of ( $k_{\text{obs}}$ ) of initial bursts were found to be linearly dependent on CDNB concentration and lacked saturation behavior, indicating less efficient binding of CDNB to the active site. Therefore, the rate of the chemical step  $k_4$  could not be obtained directly by fitting the data to Eq. 2. Instead, by using a simplified Eq. 3 for the linear relationship, the apparent second order rate  $k_4/K_d^{\text{CDNB}}$  ( $9.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) was determined, which is comparable with the  $k_{\text{cat}}/K_m^{\text{CDNB}}$  ( $7.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ), as expected. The substrate concentration at physiological conditions is expected to be lower than the  $K_m$ , and in that situation, chemistry would be the rate-limiting factor for MGST2 catalyzed reactions.

### 5.3 ONE-THIRD-OF-THE-SITES REACTIVITY OF MGST2 (PAPER III)

Kinetic and spectroscopic data show that MGST2 activates one thiolate per trimer while LTC4S uses all three monomers to form thiolate (Paper I & II). The formation of one thiolate anion per trimer has also been seen for MGST1 and mPGES1 (39,48). The primary structure of MGST2 is closely related to LTC4S and distant from MGST1 and mPGES1. The 3-

dimensional structure has been determined for all these proteins except for MGST2, and comparisons of the MAPEG members reveals a functionally active trimer as the biological unit with a GSH binding site corresponding to each monomer, which is not obvious for MGST2 and MGST3 due to the lack of structural data. The homology model of MGST2 based on the LTC4S structure (PDB ID 2UUH) suggests the key catalytic residue as Arg-104 with the residues involved in the GSH binding within the active site being highly conserved. This indicates that MGST2 mediated GSH activation could share a common mechanism with LTC4S, However, the observed third-of-the-sites reactivity for GS<sup>-</sup> formation displayed by MGST2 suggests a different mechanism compared to that of LTC4S. In this paper, more experimental evidence has been collected to investigate the catalytic mechanism of MGST2 in detail via global kinetic simulation.

Blue native-polyacrylamide gel electrophoresis (BN-PAGE) was used to estimate the native mass of the MGST2 functional unit as this technique can separate biologically active membrane protein complexes (235). Although use of non-optimal mass markers can complicate the reliable determination of molecular mass of bands, BN-PAGE has been shown to be a reliable technique for the determination of mass and oligomeric state of native membrane proteins (236). MGST2 migrated with an apparent  $M_r$  of about 57000, corresponding to a trimeric functional unit. Addition of sodium dodecyl sulfate (SDS) to the loaded sample dismantled the trimer and generated lower molecular mass bands, probably indicating the dimeric and monomeric forms. Hence, the observed quaternary structure of MGST2 indicates a homotrimeric functional unit. In addition, differential scanning calorimetry (DSC) was used to obtain independent evidence for the oligomeric state of MGST2. DSC provides indirect information about the oligomeric state of a native protein by comparing the calorimetric enthalpy ( $\Delta H^\circ_m$ ) with the Van't Hoff enthalpy ( $\Delta H^\circ_{vH}$ ). The  $\Delta H^\circ_m$  will be equal to  $\Delta H^\circ_{vH}$  if the protein unfolds as a monomer during the thermally induced transition between folded and unfolded state whereas the transition will be sharper and the ratio,  $\Delta H^\circ_{vH} / \Delta H^\circ_m$  will be more than one if the protein unfolds as an oligomer. The  $\Delta H^\circ_{vH}$  calculated from the DSC thermogram of MGST2 was about 3-fold greater than  $\Delta H^\circ_m$ , ( $\Delta H^\circ_{vH} = 146 \pm 0.7$  kcal/mol)/( $\Delta H^\circ_m = 42 \pm 0.15$  kcal/mol) = 3.5, supporting that MGST2 forms oligomers in solution. While the shape of the transition from folded to unfolded state can also be dependent on other factors, complicating the analysis of oligomeric state from the DSC results alone, these experiments together with the results from BN-PAGE provide strong evidence that MGST2 is physiologically active as a homotrimer just as LTC4S.

To further confirm the trimeric functional unit of MGST2, stoichiometry of ligand binding was determined by isothermal titration calorimetry (ITC) and equilibrium dialysis where  $\text{GSO}_3^-$  and GSH were used as ligands. The ITC experiments were performed with a 400  $\mu\text{M}$  MGST2 subunit concentration (in the cell) titrated against the successive addition of ligand from a 5 mM solution of  $\text{GSO}_3^-$  in the syringe, using 12/18 injections at 20°C. The resulting raw ITC data show negative peaks, arising from ligand binding after each injection. The amount of heat produced after each injection was then calculated from the area under the respective peaks, and plotted against the molar ratio of ligand to protein. The best fit of the binding isotherm was obtained using a one-set-of-sites model, which is described for  $n$ -identical sites. The binding parameters were calculated from the fit as binding enthalpy ( $\Delta H = -5$  kcal/mol), affinity ( $K_d^{\text{GSO}_3^-} = 36$   $\mu\text{M}$ ) and stoichiometry of ligand binding ( $N = 0.86 \pm 0.1$  per subunit). The stoichiometry (1:1 for  $\text{GSO}_3^-$ : monomer) obtained for  $\text{GSO}_3^-$  binding suggests that all three sites of the MGST2 trimer are capable of ligand binding. The affinity of GSH is comparatively low towards MGST2 (0.1-3 mM) and hence, ITC experiments could not be performed efficiently by using GSH as a ligand. However, the stoichiometry of GSH binding towards MGST2 was determined by equilibrium dialysis experiments where radiolabeled  $^{35}\text{S}$ -GSH was used to quantify the amount of GSH in each chamber after dialysis. The data were used in the global kinetic simulation and the resulting stoichiometry also corresponded to three GSH molecules per MGST2 trimer.

Global kinetic simulation was used to show that the observed third-of-the-sites reactivity for  $\text{GS}^-$  formation (Paper II) is a characteristic mechanistic feature for MGST2 catalyzed reactions. The simulation was performed using KinTek Global Kinetic Explorer (218) and the advantage of fitting kinetic data globally has already been discussed in a previous chapter (Ch. 3). The pre-steady-state kinetic data from two stopped-flow experiments, thiolate anion formation and CDNB burst, and data from equilibrium dialysis experiments, were used in the global kinetic simulation, where all the experimental traces were fitted simultaneously to a model (Scheme 3) describing the complete mechanism from substrate binding to product release. The model is simplified to represent only the productive active site of the enzyme.



Scheme 3

The rates and equilibrium constants obtained from the conventional data fitting were used as initial parameters. The observable constants as well as the extinction coefficients were defined for each individual experiment accordingly to set the appropriate parameters such as the scaling factor. The global data fitting uses the amplitude information as well as the rates for each experimental signal to achieve a good fit. The enzyme concentration used in the simulation for global data fitting process was 20  $\mu\text{M}$  subunits (describing the situation where all three sites are active) or 6.6  $\mu\text{M}$  subunits (equivalent to one productive site out of three). The best fit of the experimental data to the model was obtained by using 6.6  $\mu\text{M}$  subunit concentration of active enzyme whereas using 20  $\mu\text{M}$  subunit concentration failed to provide a reasonable fit to the model. The rates and equilibrium constants estimated by the global fit were in good agreement with those obtained from the conventional fit except for the  $K_d^{\text{GSH}}$ . FitSpace function of the KinTek explorer was used to evaluate the kinetic rates and constants by confidence contour analysis (237). However, three GSH molecules could still be bound to each trimer (based on the global simulation of equilibrium binding data) while only one forming  $\text{GS}^-$  at any one time. Thus, the global kinetic simulation conclusively demonstrates a 1/3 of the active sites reactivity for MGST2 catalyzed reactions. The 1/3 of the sites reactivity has also been shown previously for MGST1 and mPGES1, where only one active site at a time can form  $\text{GS}^-$  with the other two concurrently becoming inactive (39,48). A biological role of this mechanism could be that the inactive subunits stabilize the remaining active site upon GSH binding by conformational transition to improve catalytic efficiency (48), and that the stabilized  $\text{GS}^-$  may assist product release from the neighboring subunit (39). The crosstalk between subunits upon GSH binding could also contribute conformational change within the active site required for  $\text{GS}^-$  formation and efficient catalysis.

#### **5.4 MOLECULAR MECHANISM OF PHOSPHOREGULATION ON LTC4S ACTIVITY (PAPER IV)**

Phosphoregulation of LTC4S has been described previously where protein kinase C (PKC) mediated phosphorylation of LTC4S suppresses its activity in certain cell types. A ribosomal S6 kinase (p70S6k) has also been identified, which plays an important role in the phosphoregulation of LTC4S in monocytes (71). In this study, p70S6k phosphorylation sites were identified by an *in vitro* phosphorylation assay combined with mass spectrometry, with Ser-36 being identified as the major phosphorylation site of LTC4S. Thr-40 was also identified as being phosphorylated by the kinase with a lower frequency. The functional consequence of phosphorylation event on both these sites was investigated by site-directed

mutagenesis and kinetic analysis. Molecular dynamics (MD) simulations were performed with both phosphorylated and unphosphorylated forms to gain a mechanistic insight into the effect of phosphorylation on enzymatic structure and dynamics. In addition, the crystal structure of a phosphomimic mutant was determined to confirm its structural integrity and indicate its potential effect on the chemical mechanism via comparison with the wild type structure.

Initial prediction of potential phosphorylation site(s) was performed by using an online phosphorylation prediction tool, NetPhos 2.0 server, which identifies the sites based on sequence information. Finally, four (Ser-36, Ser-100, Thr-40 and Thr-41) out of nine potential sites were selected for experimental analysis. The residues were selected based on their probability scores and location in the structure. Residues located within the membrane lipid bilayer were excluded. The probability of these residues to be phosphorylated is very low, due to lack of accessibility of kinases through the membrane-spanning region, with integral membrane proteins most commonly possessing phosphorylation sites located within the extra-membrane loop regions.

MS/MS analysis was subsequently performed to identify the phosphorylation site(s) experimentally. The *in vitro* phosphorylated protein samples were used in the mass spectrometric analysis after trypsin digestion. The sequence coverage achieved up to 32% by MS/MS analysis and the peptide fragments containing Ser-36, Thr-40, Thr-41, and Ser-111 were identified in WT and S36A samples. Two additional mutants were used to generate detectable peptide fragments containing Ser-100 after trypsin digestion but only detected unmodified (not phosphorylated) peptides in the MS/MS analysis. The peptide fragments covering Ser-36, Thr-40, and Thr-41 were repeatedly identified as phosphorylated. Ser-36 was assigned as phosphorylated in the majority of samples as the single phosphorylation site, whereas in two instances, Thr-40 was identified as being phosphorylated instead of Ser-36. No evidence of phosphorylation at Thr-41 could be obtained.

Phosphorylation of LTC<sub>4</sub>S was also analyzed by autoradiography where an *in vitro* phosphorylation assays were performed using radioactive [ $\gamma$ -<sup>32</sup>P]ATP in the reaction mixture. It was found that phosphorylation of LTC<sub>4</sub>S is detergent specific. WT LTC<sub>4</sub>S purified using DDM as a detergent didn't show any clear radioactive band corresponding to the phosphorylated LTC<sub>4</sub>S whereas protein purified with Triton X-100 displayed a radioactive band with a size of around 18kDa. DDM has been shown crystallographically to bind to the same site as LTA<sub>4</sub> located within the inter-monomeric hydrophobic crevice of the active site (PDB ID 2UUH). The observed detergent specificity to phosphorylation could be due to

steric effects of the DDM preventing access of kinases to the potential phosphorylation site(s). No further conclusive evidence could be obtained for the assignment of phosphorylation site(s) by this method.

Phosphomimetic mutants were subsequently used to study the functional consequences of phosphorylation of LTC4S by kinetic analysis. Two mutants were prepared by replacing Ser-36 and Thr-40 with a Glu, to mimic phosphoserine and phosphothreonine, respectively. The activity of S36E (20  $\mu\text{mol}/\text{min}/\text{mg}$ ) and T40E (67  $\mu\text{mol}/\text{min}/\text{mg}$ ) mutants were reduced by nearly 80% and 30%, respectively, as compared to WT (95  $\mu\text{mol}/\text{min}/\text{mg}$ ) activity. Moreover, the steady-state kinetic parameters, obtained for those mutants by using physiological substrates LTA<sub>4</sub> and GSH, were also affected by the phosphomimetic mutations. A dramatic change was observed with the catalytic properties of the S36E mutant, where the activity became linearly dependent on LTA<sub>4</sub> concentration, indicating poor substrate binding. The decreased catalytic efficiency displayed by the S36E mutant was significant, less than 10% of WT LTC4S as judged by the apparent second order rate constant  $k_{\text{cat}}/K_m^{\text{LTA}_4}$ , which represents the rate of productive substrate binding. In contrast, the activity and catalytic properties observed for the T40E mutant were not altered significantly relative to the WT enzyme. Thus, the results obtained from kinetic analysis suggest that the introduction of a negative charge at position 36 may compromise the substrate binding and catalytic efficiency of the enzyme.

Comparative molecular dynamic simulations were performed over a 100 ns time scale using both phosphorylated and unphosphorylated forms of LTC4S embedded within a lipid bilayer. The analysis of observed structural movements during the simulation time steps indicate that the loop containing Ser-36, will move down towards the neighboring subunit of the functional homo-trimer upon phosphorylation and hence, form hydrogen bonding interactions between the phosphoserine and the catalytically important residue, Arg-104. Arg-104 is responsible for the activation and stabilization of GS<sup>-</sup> (66). Therefore, the interaction between phosphoserine and Arg-104 would lead to a reduction of LTC4S activity by disturbing the catalytic machinery as well as access to the active site.

The observed differences between the crystal structures of S36E and the WT LTC4S, such as a minor spatial shift for the thiol group of GSH and the lack of density for a DDM molecule, support the mechanism of phosphoserine inhibition suggested by MD simulations and kinetic analysis.

It has been shown in a survey that protein phosphorylation affects drug potency by inhibiting drug molecule binding to the target protein (238). In this study, phosphomimetic mutant S36E was used to check the influence of phosphorylation on inhibitor binding. Inhibition experiments were carried out with both WT and S36E LTC4S using a nano-molar inhibitor, TK04. The S36E mutant was found less sensitive ( $IC_{50}$  389±69 nM) to TK04 at low inhibitor concentrations ( $\leq IC_{50}$ ) compared to the WT enzyme ( $IC_{50}$  211±52 nM), indicating that TK04 binding was affected by phosphorylation at Ser-36. TK04 was previously proposed to occupy the LTA<sub>4</sub> binding site within the subunit interface (239). Thus, the decreased efficiency of TK04 inhibition of the S36E mutant also supports the hypothesis that lipid substrate binding is affected by phosphorylation at this site.

Therefore, phosphorylation at Ser-36 appeared functionally important for the regulation of LTC4S activity, whereas Thr-40 seemed to be an alternative site of marginal functional relevance.

## 6 CONCLUSIONS AND FUTURE PERSPECTIVE

LTC4S and MGST2 catalyze the key step during cys-LT biosynthesis, which produces a proinflammatory lipid mediator LTC<sub>4</sub>. However, both of these enzymes display distinct substrate specificity and catalytic properties. LTC4S is found to have high substrate specificity for LTA<sub>4</sub>, whereas MGST2 displays broad substrate specificity like other GSTs. The exact physiological function of MGST2 is still under debate but our results suggest that it can play roles in different types of physiological events, as a cys-LT biosynthetic enzyme or as a xenobiotic-metabolizing enzyme depending on its location in different cell types. In contrast, LTC4S has evolved specifically as a LTC<sub>4</sub> biosynthetic enzyme and thus, has specific substrate binding and catalytic properties in order to efficiently convert the unstable substrate LTA<sub>4</sub>.

In this thesis, the mechanism of GSH binding and its activation were analyzed by pre-steady-state kinetics for both LTC4S and MGST2. LTC4S was shown to have a relatively high specificity towards GSH and forms GS<sup>-</sup> rapidly by using all three monomers simultaneously in the functional homo-trimer. On the other hand, MGST2 shows one-third-of-the-sites reactivity to activate GS<sup>-</sup> as previously reported for the related enzymes, MGST1 and mPGES1. Such reactivity may play an important role in stabilizing the homo-trimer for efficient catalysis towards reactive substrates. The notion of MGST2 as functional homo-trimer is suggested by several experiments but definitive structural elucidation needs to be conducted to verify this concept. In addition, the unequivocal determination of membrane topology of the enzyme would assist in understanding its principal physiological function.

The overall catalytic mechanism of MGST2 was described in terms of electrophilic second substrates. Recharging of GS<sup>-</sup> could be a rate-limiting factor for MGST2 catalysis at low GSH concentration as seen from the rapid burst kinetics. However, the chemical step would be rate limiting for MGST2 catalyzed reactions under physiological conditions.

The phosphorylation of LTC4S at Ser-36 was identified as important for the regulation of LTC4S activity. Our results suggest that phosphorylation impairs the catalytic activity by remodeling the active site architecture and limiting substrate access. The p70S6k phosphorylation sites were identified and studied experimentally to reveal the mechanism of phosphoregulation at the molecular level. Further studies need to be performed to confirm that intracellular phosphorylation follows the same mechanism. Kinases other than p70S6k (e.g. PKC, PKA) have also been shown to mediate phosphoregulation of LTC4S in different

cell types. Therefore, new phosphorylation sites could emerge as functionally relevant along with unique mechanisms. All of these possibilities have to be explored to understand the mechanism of phosphoregulation in a given cellular context and deserve extensive investigations. .

Finally, the results presented in this thesis may also provide important clues for any future research directed towards the understanding of the mechanism and functional importance of other MAPEG members, as well as for pharmaceutical efforts to modulate their function.

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