From Institute of Environmental Medicine
Karolinska Institutet, Stockholm, Sweden

Microsomal Prostaglandin E Synthase 1 and Microsomal Glutathione Transferase 1: Inhibition and Mechanism

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Stockholm 2014

Published by Karolinska Institutet.
Printed by E-print AB

ISBN 978-91-7549-586-6

Microsomal Prostaglandin E Synthase 1 and Microsomal Glutathione Transferase 1: Inhibition and Mechanism

Thesis for Doctoral Degree (Ph.D.)

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ABSTRACT

The Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism (MAPEG) is a family of six membrane bound proteins with different biological functions. MAPEG superfamily members enable and catalyze reactions where reactive lipid intermediates are either transformed to physiological messengers or turned into unreactive compounds. Five of these proteins are enzymes that utilize glutathione in their catalytic mechanism, and two of these are the focus of this thesis.

Microsomal prostaglandin E synthase 1 (MPGES1) is induced by pro-inflammatory stimuli and is the major contributor to prostaglandin E_2 (PGE_2) synthesis during inflammation. PGE_2 mediates a number of biological responses, including the cardinal signs of inflammation by modulating vasodilation and thereby increasing blood flow, redness and swelling, as well as pain and fever. Increased biosynthesis of PGE_2 by MPGES1 has been implicated in numerous chronic inflammatory pathologies like rheumatoid arthritis and cancer. MPGES1 is therefore considered a potential drug target and has been investigated by both pharmaceutical companies and academic researchers. In collaboration with a small research and development company, we have developed inhibitors targeting MPGES1 and investigated their inhibitory mechanisms. To do that, we developed a simple medium- to high-throughput assay and concluded that these inhibitors, as well as the reference inhibitor MK-886, function mainly as competitive inhibitors towards its substrate PGH_2. We further investigated the inhibitors in rat enzyme and through site directed mutagenesis found that the cleft where the inhibitors enter, and presumably also PGH_2 enters, has more steric hindrance in the rat enzyme compared to the human enzyme. This discovery enlightened the reason why several potent human MPGES1 inhibitors didn't have any effect in rodent animal models. Furthermore, we developed dual MPGES1 inhibitors for human and rat enzyme and characterized those in *in vitro* assays and in *in vivo* rodent models of inflammation. *In vivo* we compared the prostanoid profile after pharmacological inhibition to that after genetic deletion of MPGES1. Differences in the effect of MPGES1 pharmacological inhibition and genetic deletion were detected, as well as differences between different models. We conclude thereby that it is important to compare different inhibitors in the same models with similar conditions, in order to have a significant comparison, and to complement results from knockout animals with inhibition results in wild type systems.

Microsomal glutathione transferase 1 (MGST1) is a membrane bound glutathione transferase that is involved in cellular protection from oxidative stress and xenobiotics. MGST1 catalyzes conjugation reactions of glutathione to reactive (electrophilic substrates) so they can be more readily excreted from the cells. The enzyme displays broad substrate specificity and is most prevalent in the liver, which is the place where the most important part of drug metabolism occurs. A unique and very interesting feature of this glutathione transferase is that it can be activated by modification with sulfhydryl reagents and proteolysis. Our group has conducted extensive research on MGST1 and uncovered several aspects of its catalytic mechanism through both steady-state kinetics and pre steady-state kinetic experiments. We have now

concluded all the information about the microscopic steps of the enzyme's global kinetic mechanism and derived the steady-state rate expression. By comparing calculated catalytic constants to our experimental values we have discovered a pre-existing resting state to this enzyme which is most pronounced at low temperatures and low reactive electrophilic substrates. We propose that limited turnover pre steady state experiments are the best way to understand the physiologically relevant catalytic mechanism of MGST1.

In conclusion, this thesis provides a deeper understanding of two important members of the MAPEG superfamily of enzymes with different physiological functions and catalytic mechanisms of action. We have gained insights into the structure and inhibitory mechanisms of MPGES1 as well as characterized potent inhibitors of this enzyme in models of inflammation. Our findings constitute new tools in the study of MPGES1. We have also unraveled the global mechanism of MGST1 which is the closest MAPEG member to MPGES1 based on sequence similarity. Our methods used for the determination of the catalytic mechanism of MSGT1 have the potential to assist in defining the catalytic mechanism of MPGES1.

POPULAR SCIENCE SUMMARY

Some of you reading this thesis may have heard about oxidants, but many more are probably aware of the beneficial effects that we seek from antioxidants - in e.g. fruit and vegetables and in expensive lotions. Oxidants in biological systems can cause harmful reactions involving the membrane bound polyunsaturated fatty acids in the cells, to give an example. In this thesis, one of the body's most important antioxidants is presented, glutathione. Glutathione participates in an important part of the body's defense against oxidants. Our research focuses on a protein that specifically uses glutathione to defend the body against compounds that cause, and are formed during, oxidation. This protein is called MGST1. Our research makes it possible to determine the exact mechanism of how MGST1 and glutathione collaborate when they put a stop to the harmful oxidative processes. We have shown through mathematical calculations (equations) which steps in the process are fast or slow. By comparing the calculated with the experimental values, we have been able to determine the complete mechanism for MGST1. This is important information for us to gain a better understanding of the body's defense mechanisms, and to understand how drugs are digested by the body, or why chemotherapy doesn’t work in the treatment of some cancers.

However, many oxidative processes in the cell membrane are advantageous. Organisms have through evolution learned to turn external threats into signaling systems in cells. Production of short-range acting hormones, called prostaglandins, is an example of this. Prostaglandins are produced through oxidation by a group of proteins that can be found in cell membranes. Prostaglandins act in the protection of gastrointestinal integrity and the transmission of fever and pain. One of the membrane proteins involved is MPGES1, which is part of the production of prostaglandin E_2. Prostaglandin E_2 is a signal molecule secreted from cells during inflammation and in e.g. rheumatoid arthritis and cancer. Today, there are numerous anti-inflammatory drugs such as Aspirin, Diclofenac and Ibuprofen. These drugs act by indirectly preventing the formation of prostaglandin E_2 but can also cause adverse effects. The most common adverse effects among anti-inflammatory drugs are gastric ulcers although they also increase the risk of cardiovascular diseases. Therefore it is important to develop drugs that prevent inflammation by stopping only MPGES1, without affecting other signaling pathways in the body and thereby circumvent the side effects. Our research has focused on finding effective inhibitors of MPGES1 and investigation of the exact way in which these inhibitors interact with the protein. We have also found differences between the human protein and the protein from rat. It is important to have knowledge about such differences, as drugs are tested in animal models, where rat models play a very important role. We have further examined whether these inhibitors interact with other proteins and finally tested them in animal models of inflammation. The inhibitors do have a positive effect, which is important knowledge when developing them into a drug for human use.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Några av er som läser denna avhandling har kanske hört talas om oxidanter, men ännu fler är nog medvetna om de gynnsamma effekterna som antioxidanter tros förmedla – bl.a. i frukt och grönt och i dyra hudkrämer. Oxidanter i biologiska system kan skapa skadliga reaktioner för t.ex. de bundna fleromättade fettsyrorna i cellernas membran. I denna avhandling kommer en av kroppens viktigaste antioxidanter att presenteras, glutation. Glutation deltar i en viktig del av kroppens försvar mot oxidanter. Vår forskning fokuserar på ett protein som specifikt använder glutation för att försvara kroppens membran mot substanserna som orsakar oxidation eller bildas under oxidativa processer. Detta protein kallas MGST1. Vi har visat att det är möjligt att bestämma den exakta mekanismen för hur MGST1 och glutation samarbetar när de sätter stopp för de skadliga oxidativa processerna. Vi har tagit fram matematiska beräkningar (ekvationer) som beskriver vilka steg i processen som är snabba och långsamma. Genom att jämföra beräkningarna med experimentella värden har vi kunnat visa den fullständiga mekanismen för MGST1. Detta är viktigt för att vi bättre ska förstå kroppens skyddsmekanismer och för att förstå hur ett läkemedel bryts ned av kroppen eller varför en del cellgifter (cytostatika) inte fungerar vid cancerbehandling.

Förutom de skadliga finns det även många gynnsamma oxidativa processer i cellernas membran. Under evolutionens gång har organismer lärt sig att använda dessa oxiderade membrankomponenter till att skapa signaleringssystem i cellerna. Ett sådant exempel är kroppens tillverkning av kortdistansverkande hormoner, så kallade prostaglandiner. Prostaglandiner tillverkas via oxidation av en grupp proteiner som finns i cellernas membran. Prostaglandiner har till uppgift att bl.a. skydda slemhinnor i mag-tarmkanalen men även ge upphov till feber och smärta. Ett utav proteinerna i cellmembranet är MPGES1 som medverkar i produktionen av prostaglandin E_2. Prostaglandin E_2 är en signalmolekyl som utsöndras från kroppens celler i samband med inflammation och vid t.ex. ledgångsreumatism och cancer. Idag finns åtskilliga antiinflammatoriska läkemedel, Magnecyl, Voltaren och Ipren för att nämna några. De verkar genom att indirekt hindra bildning av prostaglandin E_2 men de har även skadliga effekter. De vanligaste biverkningarna bland antiinflammatoriska läkemedel är magsår men de ökar även risken för hjärtsjukdomar. Det är därför viktigt att utveckla läkemedel som förhindrar inflammation genom att enbart stoppa MPGES1 men utan att påverka andra signalvägar i kroppen och därmed undvika biverkningar. Vi har i vår forskning inriktat oss på att hitta hämmare till MPGES1 och till att undersöka exakt hur dessa hämmare samspelar med proteinet. Vi har vidare hittat skillnader mellan det mänskliga proteinet och proteinet hos råtta. Det är viktigt att ha kunskap om sådana skillnader då läkemedel behöver testas i djurmodeller, där råttmodeller är mycket vanliga. Vi har även undersökt om dessa MPGES1 hämmare interagerar med andra proteiner och slutligen även testat dem i djurmodeller. Hämmarna har positiva effekter vilket är viktig kunskap när man ska utveckla dem till läkemedel.

LIST OF SCIENTIFIC PAPERS

I. **Identification of Key Residues Determining Species Differences in Inhibitor Binding of Microsomal Prostaglandin E Synthase-1**
Pawelzik SC, Uda NR, **Spahiu L**, Jegerschöld C, Stenberg P, Hebert H, Morgenstern R, Jakobsson PJ
J Biol Chem. 2010; 285 (38): 29254-61

II. **A Facilitated Approach to Evaluate the Inhibitor Mode and Potency of Compounds Targeting Microsomal Prostaglandin E Synthase-1**
Spahiu L, Stenberg P, Larsson C, Wannberg J, Alterman M, Kull B, Nekhotiaeva N, Morgenstern R
Assay Drug Dev Technol. 2011; 9 (5): 487-95

III. **Characterization of a Human and Murine mPGES-1 Inhibitor and Comparison to mPGES-1 Genetic Deletion in Mouse Models of Inflammation**
Leclerc P, Idborg H, **Spahiu L**, Larsson C, Nekhotiaeva N, Wannberg J, Stenberg P, Korotkova M, Jakobsson PJ
Prostaglandins Other Lipid Mediat. 2013; 107: 26-34

IV. **Global Kinetic Mechanism of Microsomal Glutathione Transferase 1**
Spahiu L, Ålander J, Svensson R, Lehmer C, Armstrong R, Morgenstern R
Manuscript

PAPERS NOT INCLUDED IN THESIS

Characterization of a New mPGES-1 Inhibitor in Rat Models of Inflammation
Leclerc P, Pawelzik SC, Idborg H, **Spahiu L**, Larsson C, Stenberg P, Korotkova M, Jakobsson PJ
Prostaglandins Other Lipid Mediat. 2013; 102-103: 1-12

CONTENTS

LIST OF ABBREVIATIONS

12-HHT	12-hydroxyheptadecatrienoic acid
2,5-DCNB	2,5-dichloronitrobenzene
5-LO	5-lipooxygenase
AA	arachidonic acid
CDNB	1-chloro-2,4-dinitrobenzene
COX	cyclooxygenase
COXib	selective COX2 inhibitor
cPGES	cytosolic prostaglandin E synthase
$cPLA_2$	cytosolic phospholipase A_2
cysLT	cysteinyl leukotriene
EIA	enzyme immunoassay
ER	endoplasmic reticulum
FLAP	5-lipoxygenase activating protein
GPCR	G-protein coupled receptor
GPX	glutathione peroxidases
GSH	glutathione (reduced)
GSSG	glutathione disulfide (oxidized)
GST	glutathione transferase
HPGDS	haematopoietic prostaglandin D synthase
LO	lipoxygenase
LPGDS	lipocalin-type prostaglandin D synthase
LT	leukotreine
LTC_4S	leukotreine C_4 synthase
MDA	malondialdehyde
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MGST	microsomal glutathione transferase
MPGES	microsomal prostaglandin E synthase
NADPH	nicotinamide adenine dinucleotide (reduced)
NSAID	non-steroidal anti-inflammatory drug

PG	prostaglandin
PGFS	prostaglandin F synthase
PGHS	prostaglandin H synthase
PGIS	prostaglandin I synthase (prostacyclin synthase)
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
RP-HPLC	reverse-phase high-performance liquid chromatography
RX	reactive substrates
TBA	thiobarbituric acid
TX	thromboxane
TXS	thromboxane synthase

1 INTRODUCTION, CHAPTER 1

1.1 MEMBRANE PEROXIDATION

Structurally, the cell membrane is the physical barrier that encloses the cell from its outer environment. It provides protection in many different ways and is primarily constituted by lipids and proteins. Apart from the external cell membrane, eukaryotic cells have membranes that enclose their organelles, such as the endoplasmic reticulum (ER), Golgi apparatus, mitochondria and nucleus. The main lipids of all eukaryotic membranes are the phospholipids which are amphipathic molecules with both a hydrophobic and a hydrophilic part. The hydrophobic part of phospholipids consists of 14 to 24 carbon long fatty acids which are either saturated or can contain one or more double bonds. The fluidity of the membranes is dictated by the amount of polyunsaturated fatty acids (PUFA) such as linoleic, linolenic and arachidonic acid [1]. For each additional double bond, which normally is in its *cis* configuration, the melting point of the fatty acid is lowered as their arrangement in the phospholipid bilayer weakens the hydrophobic interactions of the hydrocarbons. The melting point is also affected by the length of the fatty acid; shorter chains give lower melting points. The fluidity of membranes influences the diffusion of water and lipophilic molecules though the lipid bilayer.

Molecular oxygen is crucial for aerobic life, but its beneficial properties come with a price. The reduction of oxygen is mostly realized in the mitochondrion, considered to be the cellular "power house". This route also induces formation of reactive oxygen species (ROS) such as superoxide radical ($O_2^- \cdot$) and hydrogen peroxide (H_2O_2), all with potentially damaging properties for many biological structures. Low levels of ROS are normally required for many redox-dependent cellular processes [2]. An imbalance in ROS flux and antioxidant capacity creates oxidative stress. ROS and oxidative stress have, since free radicals were first discovered in biological materials 60 years ago, repeatedly been associated with cell damage, cancer, and ageing [3]. PUFA of membrane lipids are particularly vulnerable to attacks from free radicals created during oxidative stress. Free radicals have the ability to react with PUFA structures, ultimately damaging the integrity of the membranes - a process called lipid peroxidation. One of the main harms of this process is that it can propagate quickly and even more free radicals are created through metal ion (mainly Fe^{2+}) enabled chain reactions and e.g. breakdown of H_2O_2 [4]. Lipid peroxidation of cell membranes can also be caused by metabolism of xenobiotics that are lipophilic and accumulate in membranes. Xenobiotics are foreign chemical compounds such as drugs, polycyclic aromatic hydrocarbons or even natural substances not normally occurring in the cell. They can cause lipid peroxidation directly through increased free radical generation or through inhibition of the cellular defense systems.

Oxidative stress and lipid peroxidation are not common in the cells normal state as eukaryotic cells have developed a series of cellular defenses to protect themselves from the damaging properties of endogenous and foreign reactive compounds. Antioxidants form an important

part of the cellular defense strategy by allowing the free radicals to abstract hydrogen atoms from their structure resulting in a lesser reactive species [1]. α-Tocopherol (vitamin E) and β-carotene (pro-vitamin A) are antioxidants and normal components of cellular membranes. Cells also employ enzymes in their membrane defense against xenobiotics, a pathway that will be further elaborated in the coming sections.

However, there are also beneficial functions of lipid peroxidation and one of them will be discussed in detail throughout this thesis: prostaglandin biosynthesis.

1.2 GLUTATHIONE

Glutathione (GSH) is a small tripeptide and the most abundant intracellular thiol source in eukaryotic cells. It is a pivotal nucleophile and reducing agent in protective metabolism and redox regulation and is consequently abundant in the liver, which is the body's "detoxification center". Peptide links and cysteine containing molecules are readily degraded in the cellular environment, but GSH is not susceptible to the action of cellular peptidases thanks to its distinguishing structure. It is composed of glutamate, cysteine and glycine and has two characteristic structural features which promote its intracellular stability and diverse functions in the cell: the γ-glutamyl linkage and the cysteine thiol (Figure 1) [5, 6].

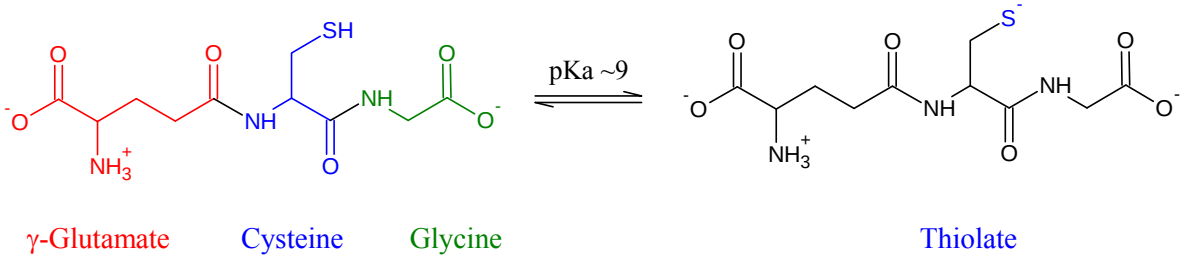


Figure 1. Glutathione is a potent nucleophile, through its ability to deprotonate its cysteine moiety thiol to a thiolate anion, and also a very versatile biological reducing agent.

Thiol biochemistry is essential to many cellular reactions as thiols are easily oxidized and rapidly regenerated. GSH can be found in almost all animal cells at rather high intracellular concentrations, ranging from 0.1 to 15 mM [7]. Constant regeneration of GSH occurs in the cells mainly through *de novo* synthesis by the joint actions of glutamate-cysteine ligase and glutathione synthase [6]. GSH is also recycled through the reduction of its oxidized form, glutathione disulfide (GSSG), by glutathione reductase and NADPH [8]. Most cells have much lower concentration of GSSG than GSH, it being approximately 100 times lower than the GSH content [1, 7].

$$GSSG + NADPH + H^+ \xrightarrow{\text{glutathione reductase}} NADP^+ + 2GSH \qquad (1)$$

The three main physiological functions of this endogenous small molecular weight thiol are redox reactions, cellular defense against oxidative stress, xenobiotic and eicosanoid

metabolism, and signal transduction [9]. Glutathione also functions as a storage and transport form of cysteine. GSH is an important cellular antioxidant which protects cells directly or indirectly from the toxic effects of ROS and lipid peroxides generated in membrane oxidation. Regulation of the intracellular redox state by GSH is maintained mainly through the actions of glutathione peroxidases (GPX) and peroxiredoxin which yields GSSG [10, 11].

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG$$

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

GSH dependent systems are the most vital defense against lipid peroxidation in mammalian cells. GSH is involved in cellular protection against lipid peroxidation caused by xenobiotics through both non-enzymatic and enzymatic pathways and is an essential cofactor in prostaglandin biosynthesis.

1.3 ENZYMATIC MEMBRANE PROTECTION

Cellular membranes have both integral and membrane associated proteins which are responsible for a wide variety of functions, including transport, defense, communication and biosynthesis. The enzymes discussed in this thesis are integral membrane proteins which utilize GSH and have different biological functions.

Enzymatic conjugation of GSH to different electrophilic compounds in rat liver extracts was linked to mercapturic acid metabolism in the early 1960's [12, 13]. This was the starting point of several decades of investigation on the activity of glutathione transferases (GSTs) and their ability to catalyze conjugation reactions to electrophilic carbon, nitrogen, or sulphur atoms of a large variety of reactive substrates (RX) [7, 14-16]. These reactions often result in a decreased toxicity and increased solubility of the electrophiles, which, when conjugated to GSH, can be further metabolized (in mercapturic acid metabolism) and excreted. GSTs are by this mechanism involved in the detoxification of numerous carcinogenic, mutagenic, toxic and pharmacologically active compounds [17].

$$GSH + RX \xrightarrow{GST} GSR + HX$$

GSTs are mainly localized in the cytosol and it was thus the cytosolic (or soluble) enzymes which were the first to be characterized. Mammalian GST nomenclature, based on sequence similarities, immunological cross reactivity and structural and kinetic properties [18, 19], has divided the mammalian enzymes into the following subclasses: Alpha (A), Kappa (K), Mu (M), Omega (O), Pi (P), Sigma (S), Theta (T) and Zeta (Z). The cytosolic GSTs belong to a widespread superfamily with a constantly increasing number of members and subclasses. This superfamily is believed to have evolved from an ancient common ancestor as a defense system to oxidative stress and throughout evolution new members evolved through a combination of gene amplification and divergence [19, 20]. Even though they share limited

sequence similarity, the GST fold is preserved throughout the different classes [14, 16, 19]. A very important aspect of GST catalysis is the ability to lower the pK_a of GSH from 9 to approximately 6. Several amino acids in the active site contribute to the stabilization of the thiolate anion GS^- [21]. GSTs constitute 3% of human liver protein [1]. Even though this superfamily of enzymes has been intensely studied, little is known about the enzymes biological functions and their natural substrates, as mainly synthetic compounds have been used to assay GSTs. Recently, efforts have been made to better characterize, classify, and determine the function of this large enzyme family [22].

GST expression is up-regulated in response to oxidative stress [23], and their importance to the cellular defense system is exemplified by their tremendous excess in the liver. It has been calculated that these enzymes can deplete the liver GSH pool in a matter of seconds provided there are large amounts of reactive substrates to use in the conjugation reactions. Depletion of GSH reservoirs is accompanied by severe toxicity [24] and despite the excess of GSTs in the liver, their main activity is resting. GST overcapacity can be illustrated by the fact that their actual turnover rate is approximately one turnover per enzyme molecule every second day [25].

GSH and GSTs have also been suggested to protect tumors from chemotherapy [26]. The great variety and substrate promiscuity of GSTs has also inspired basic research to study fundamental issues of enzyme evolution and function [16, 27-29].

One of the GSTs involved in the detoxification process against xenobiotics acts specifically in membranes; the microsomal GST 1 (MGST1) [30-32]. Similar to many cytosolic GSTs, MGST1 has a broad substrate specificity [33] and is most prevalent in the liver, where the most important part of drug metabolism occurs [34]. In rat liver cells it constitutes 3% of the ER protein and 5% of the outer mitochondrial membrane [35]. Studies of this protein have been ongoing since it was first discovered in 1979 [30-34, 36]. A unique feature of this GST is that it can be activated, up to 30-fold, by modification with sulfhydryl reagents and proteolysis [30, 31, 37].

1.4 REACTIVE LIPID MEDIATORS

Not all lipid peroxidation processes in mammalian cells are deleterious. As explained above, ROS formation in membranes is a natural process that occurs frequently and evolutionary adaptation has led to the development of several defense mechanisms against oxidative stress. Living organisms have simultaneously learned to advantageously make use of chemical reactivity and developed physiological functions dependent on ROS. A mild oxidative state is vital for cell survival, where ROS act as inducers of various biological functions and play a key role as mediators of numerous signaling processes [38]. By such mechanisms, lipid peroxidation at moderate levels has an important function in cellular signaling, and is driven by either non-enzymatic or enzyme-catalyzed reactions. As oxidative stress is closely related

to many pathological conditions, it is evident that the beneficial and detrimental functions of ROS need to be delicately balanced for life to prosper.

The essential fatty acid arachidonic acid (AA) is one of the most common PUFAs in the phospholipids of mammalian cell membranes (17% in membranes from rat liver microsomal fractions [1]) and plays an important role as the main precursor in prostaglandin (PG) biosynthesis [39, 40]. PG biosynthesis is a process where natural lipid peroxides are generated. These natural lipid peroxides are mediators (signaling molecules, some of them reactive) which are synthesized *de novo* by most mammalian cell types and through their actions as local hormones [41] they regulate a number of (patho-)physiological processes, including allergic [42] and inflammatory responses [43], cardiovascular control [44], and platelet aggregation [45].

It was as late as the year 1990 that a non-enzymatic pathway for the formation of PG derivatives *in vivo* was described by a mechanism involving free radical-catalyzed oxidation of AA [46]. The discovery of isoprostanes, which are PG-like compounds, showed that potent lipid mediators are produced *in vivo* non-enzymatically in oxidation reactions initiated by free radicals [47]. Other non-enzymatically derived PG derivatives have also been identified and it is very possible that this family of compounds may include more identified members in the future. Isoprostanes have been intensely characterized during the past two decades and have been shown to be reliable biomarkers of oxidative stress [48]. Their validity as biomarkers and association to different human diseases needs to be further characterized [49] and it would be interesting to raise the question whether these compounds are in fact PG ancestors. The non-enzymatically produced mediators could have gained biological significance in mammalian cells and thus enzymatic pathways of PG synthesis would have arisen through evolution.

PG biosynthesis is primarily realized enzymatically and initiated by the activity of two PGH_2 synthases (PGHS): PGHS1 and PGHS2. These two isozymes catalyze the oxidation of AA into the unstable endoperoxides PGG_2 and PGH_2, and PGH_2 is in turn further isomerized by terminal enzymes of the PG cascade. PGHS1 and PGHS2 are membrane associated enzymes, prevalent in the ER and nuclear membrane, which exhibit both cyclooxygenase and peroxidase activity and are thus also known as cyclooxygenases (COX1 and COX2) [39, 50]. Interestingly, the cyclooxygenase activity in both COX1 and COX2 is peroxide-dependent [51, 52], and peroxides are thus required for the activation and sustained activity of these enzymes. COX1 requires higher concentrations of peroxide for activation and sustained activity and thus must have a higher turnover of PGG_2 than COX2 [53]. Furthermore, the cyclooxygenase activity of COX1 and COX2 is also modulated by fatty acids, which can be both substrates and non-substrates to the enzymes [54].

2 BACKGROUND, CHAPTER 2

2.1 EICOSANOIDS

Eicosanoids are biologically active, 20 carbon fatty acid derivatives produced either via the COX pathway or the lipoxygenase (LO) pathway [39-41]. The COX pathway generates prostanoids, including PGs and thromboxane, whereas the LO pathway generates leukotrienes (LTs). PGs and LTs have profound physiological effects at low concentrations, but differ from global hormones by having a very short half-life and are therefore not transported via the circulation, presenting essentially a local effect. Eicosanoids are produced by most mammalian cell types and interact with individual receptors, most of which form part of the G-protein coupled receptor (GPCR) family [55], exhibiting through them a wide range of biological functions. Interestingly, the effects of these lipid mediators can vary depending on cell type.

AA is stored in membranes where its levels are carefully regulated. Eicosanoid biosynthesis is initiated by release of AA by phospholipases. The principal phospholipase in the initiation of eicosanoid biosynthesis is the cytosolic phospholipase A_2 ($cPLA_2$) [56]. Any stimuli that leads to an increase of intracellular Ca^{2+} levels, induces translocation of $cPLA_2$ to the membrane and triggers hydrolysis of AA from cellular membranes [57]. After its release AA is rapidly oxidized by a host of enzymes to yield different eicosanoids, the specific type being determined by the set of enzymes expressed at the site of production.

2.1.1 Lipoxygenase pathway

Through the activation of 5-lipoxygenase (5-LO) by 5-lipoxygenase activating protein (FLAP) [58-60], LTs are formed from AA. LTA_4 is an unstable epoxide which, like PGH_2 in the COX pathway, is the precursor of all LTs. LTs are potent pro-inflammatory mediators formed primarily by inflammatory cells and crucial in the pathogenesis of respiratory diseases and allergic reactions [61]. Several pharmaceutical treatments which interfere with LT biosynthesis entered into clinical practice for asthma therapy at the end of the last century [62, 63].

LTC_4 synthase (LTC_4S) catalyzes the specific conjugation of LTA_4 to GSH [64], leading to the formation of LTC_4, which is further metabolized to LTD_4 and LTE_4 [61]. These three LTs are denoted cysteinyl LTs (cysLTs) and are potent mediators of asthma and act through the receptors cysLT1 (specifically LTD_4) and cysLT2 (specifically LTC_4) [65]. LTC_4S deficient mice are resistant to asthmatic challenge, as is the case for the enzymes higher up the cysLT cascade [66, 67].

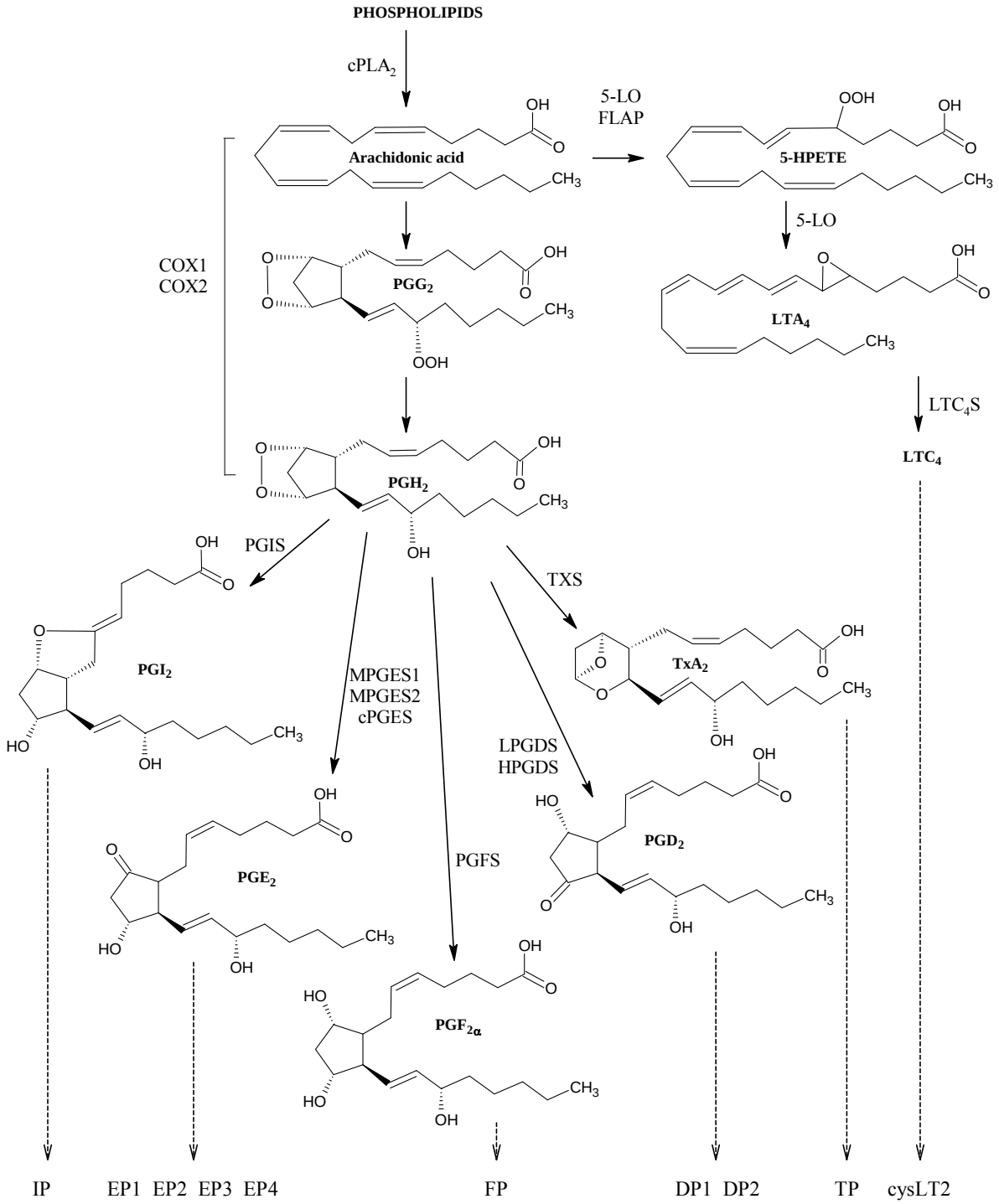


Figure 2. Biosynthetic cascade of prostaglandins (PGs) and leukotrienes (LTs), including the precursor arachidonic acid, all intermediates, products, catalyzing enzymes and receptors identified.

2.1.2 Cyclooxygenase pathway

Eicosanoid formation through the COX pathway can be either constitutive (COX1) or induced by pro-inflammatory stimuli (COX2), both generating the PG precursor PGH_2. PGH_2 is very unstable [68, 69] and is rapidly converted to PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) or thromboxane (TX) A_2 in a cellular environment by the actions of terminal isomerases/synthases of the PG cascade. These lipid mediators are mutually referred to as prostanoids.

PGD_2 is involved in the regulation of sleep [70, 71] and moreover acts as a mediator of allergy and inflammation [42]. Lipocalin-type PGD synthase (LPGDS) and haematopoietic PGD [72] synthase (HPGDS) [73, 74] are two rather different enzymes, in terms of structure, cellular distribution and evolutionary origin, which catalyze the isomerization reaction of PGH_2 into PGD_2 [75, 76]. LPGDS is a secretory protein [76] expressed in the central nervous system [77], male genital organs [78] and human heart [79]. HPGDS is a sigma class cytosolic GST [80]. The signal transduction system involving PGD_2 acts through two distinct receptors, namely DP1 and DP2, of which only DP1 is a GPCR [81, 82].

The actions of $PGF_{2\alpha}$, which initiates parturition in mammals [83] and induces potent smooth muscle contraction in the human uterus [84], are mediated via the $PGF_{2\alpha}$ receptor FP [85]. PGF synthase (PGFS) catalyzes the reduction reaction of PGH_2 into $PGF_{2\alpha}$, but $PGF_{2\alpha}$ can also be formed through the actions of PGE_2 9-keto reductase on PGE_2 [44]. $PGF_{2\alpha}$ is a stable molecule in aqueous solution, but has a very short half-life *in vivo* [86].

PGI_2 and TXA_2 have an important role in cardiovascular physiology and exhibit often opposing effects. These two eicosanoids are therefore regarded as significant regulators of the cardiovascular system. Both PGI_2 and TXA_2 are unstable and rapidly degraded, in a non-enzymatic manner, to their respective metabolites, 6-keto $PGF_{1\alpha}$ and TXB_2, by hydrolysis in aqueous solution at 37˚C [87, 88]. PGI_2 is a potent inhibitor of platelet aggregation [89] and a powerful vasodilator [90] and binds to the specific IP GPCR [91]. TXA_2 functions through two isoforms (α and β) of the TP GPCR that differ in cellular distribution and are involved in platelet aggregation and vasoconstriction [92]. Prostacyclin synthase (PGIS) [93] catalyzes the isomerization of PGH_2 to PGI_2 and thromboxane synthase (TXS) catalyzes the endoperoxide's conversion to TXA_2 [88]. Both PGIS and TXS have been shown to belong to the cytochrome P450 superfamily of enzymes [94, 95].

2.1.2.1 PGE_2 production

PGE_2 is a pleiotropic and extensively investigated eicosanoid which has an important role as mediator of inflammation in chronic inflammatory diseases. PGE_2 mediates a number of biological responses through the GPCRs EP1, EP2, EP3 and EP4 which all belong to the same subfamily of GPCRs [96]. One of the main focus areas involving PGE_2 has been its ability to regulate the cardinal signs of inflammation. PGE_2 mediates vasodilation and thereby increases blood flow, redness and swelling [97, 98], as well as pain hypersensitization [99] and fever [100] at sites of inflammation.

PGE_2 is a relatively stable molecule in aqueous solution *in vitro*, but is rapidly degraded *in vivo* and converted to an inactive metabolite (13, 14-dihydro-15-keto PGE_2) by the prostaglandin 15-dehydrogenase pathway [101]. The half-life of PGE_2 in blood is less than a minute and normal its concentration in plasma is 3-12 pg/ml [102].

There exists three distinct PGE_2 synthases. One is cytosolic prostaglandin E synthase (cPGES) and two are membrane bond (microsomal prostaglandin E synthase 1 and 2; MPGES1 and MPGES2). MPGES1 is the major contributor to PGE_2 synthesis during inflammation [103]. The inducible enzyme MPGES1 was discovered by our group together with our collaborators in 1999 [104]. MPGES1 is associated with a number of different pathophysiological states like chronic inflammation and cancer. Similar to the expression of COX2, MPGES1 is up-regulated under pro-inflammatory conditions *in vitro* [105, 106]. Moreover, COX2 and MPGES1 are predominantly co-expressed and functionally coupled to generate an outburst of PGE_2 under inflammatory conditions [107].

2.1.3 MPGES1 as pharmaceutical target

Salicylic acid was the first commercially produced non-steroidal anti-inflammatory drug (NSAID) by the Kolbe company in Germany in 1860. An improved form, acetylsalicylic acid, was introduced to the market by the pharmaceutical company Bayer in 1899 [108] and is still a worldwide used drug. It wasn't until the 1970's that its mechanism of action was discovered as the inhibition of prostaglandin synthesis through the cyclooxygenase pathway [109]. Classical NSAIDs, which inhibited both COX1 and COX2, were soon discovered to cause severe gastric ulcerations as side effects of chronic use [110]. After the discovery of the inducible COX2, selective COX2 inhibitors (COXibs) were developed to prevent inflammation and pain while disturbing less the gastric mucosa integrity due to reduced COX-1 inhibitory activity [111, 112]. However, randomized and placebo-controlled trials demonstrated an increased cardiovascular risk in patients after long-term use of selective COX2 inhibitors [113, 114]. Such an effect was also demonstrated for traditional NSAIDs [115], and led eventually to the withdrawal of Merck's Vioxx (rofecoxib) from the market [116].

The release of chemical mediators during chronic inflammation is variable in onset and duration, as chronic inflammations is a dynamic process including different phases, depending on the status of the disease. PGE_2 is one of the major pro-inflammatory PGs derived from the inducible COX2. MPGES1 can be induced in concord with COX2 by pro-inflammatory stimuli in animal models of inflammation [117, 118]. In addition, genetic deletion of MPGES1 in mice renders them resistant to fever and chronic inflammation [119, 120] similarly to COX2 deletion [121, 122]. These MPGES1 deficient mice lose their capacity to induce PGE_2 biosynthesis above normal levels and have a strongly reduced inflammatory response. Moreover, pharmacological inhibition of MPGES1 alleviates inflammatory symptoms in *in vivo* models [123]. Increased biosynthesis of PGE_2 by MPGES1 has been implicated in numerous chronic inflammatory pathologies. High amounts of PGE_2 and expression of the inducible enzymes of this pathway are found in rheumatoid

arthritis patient samples [124, 125]. Elevated levels of MPGES1 and COX are found in a number of cancers and PGE_2 modulates the inflammatory microenvironment and tumor immunosuppression which creates favorable conditions for tumor progression [126]. Furthermore, MPGES1 induction has been correlated to poor prognosis in colorectal neoplasms [127].

Drugs targeting MPGES1 are expected to have fewer adverse effects by better maintaining the gastric mucosa integrity compared to traditional NSAIDs and by avoiding increased incidence of severe cardiovascular side effects related to COXibs. Several international pharmaceutical companies have investigated MPGES1 as a drug target. Merck has reported the characterization of this enzyme [128-130] and developed several potent inhibitors [123, 131-133], the most well characterized being MF63 [123]. Pfizer has also investigated MPGES1 as a potential drug target through animal models [120, 134-137] and developed several potent inhibitors of this enzyme [138-142]. AstraZeneca recently released an X-ray crystal structure at 1.2 Å resolution [143], reported a high throughput assay for MPGES1 [144] and published the characterization of inhibitors [145, 146].

2.2 MAPEG SUPERFAMILY OF ENZYMES

The Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism (MAPEG) [147] superfamily has been attributed a somewhat misleading name as it projects an image of proteins which simply are associated to membranes instead of, as is the case, integral membrane proteins. It includes six proteins, the already introduced MGST1, MPGES1, LTC_4S, FLAP and two additional membrane bound GSTs, MGST2 [148] and MGST3 [149]. MAPEG family members enable and catalyze reactions where reactive lipid intermediates are either transformed to physiological messengers (MPGES1, LTC_4S, FLAP) or turned into unreactive compounds (MGST1-3).

The nomenclature for microsomal prostaglandin E synthase has varied throughout the years. The most commonly used abbreviation is mPGES-1 [104], although also mPGES1 and MPGES1 have been used [150, 151]. A lowercase letter preceding the protein's name abbreviation normally indicates species, where "m" represents a mouse homologue, "h" a human homologue, and so forth for each relevant species. The lowercase "m" used in the abbreviation mPGES-1/mPGES1 does not refer to the murine protein but merely to the fact that this is a membrane bound protein. The use of a lowercase letters initiating the abbreviation can sometimes be observed for other members of the MAPEG superfamily, such as the two prostaglandin D synthases LPGDS ("l"PGDS) and HPGDS ("h"PGDS), whereas it is not used in the nomenclature of the glutathione transferases belonging to this family. To simplify matters, MPGES1 will be used throughout this thesis as the only abbreviation for the enzyme microsomal prostaglandin E synthase 1.

2.2.1 MGST1 as a pharmaceutical target

MGST1 is a 17.3 kDa homo-trimeric enzyme localized mainly at the ER and mitochondrial membrane [35] and constitutes 1% of the endoplasmic reticulum protein in human liver [152]. Its substrates are very hydrophobic, reactive molecules which are often localized at cellular membranes [37, 153, 154]. This enzyme is thus involved in the biotransformation of membrane-embedded reactive electrophiles. By this mechanism, MGST1 protects cells from lipid peroxidation in the membranes and oxidative stress [155-157]. MGST1 also functions in drug metabolism by conjugation of the xenobiotics to GSH, as well as in the biotransformation of glyceryl trinitrate [25, 158].

Glutathione peroxidases can protect from lipid peroxidation in cellular membranes [16, 26] and thereby reduce the toxic effects which originate from oxidative stress. MGST1 displays peroxidase activity [159]. MGST1 expression has been reported to be both up- and down-regulated in treatments causing oxidative stress and is up-regulated in a wide variety of tumors [152]. MGST1 has also been reported to protect tumors from cytostatic drug treatment [160] and could therefore be a viable anti-cancer drug target [161].

2.3 ENZYMOLOGY

2.3.1 Steady-State Kinetics

Initial characterization of enzymes is often based upon steady-state kinetics as this method is the most commonly used means of investigating enzyme-catalyzed reactions [162]. The methodology is based on the principle that the concentration of enzyme-substrate intermediate(s) remains constant by having equal rates of formation and decay.

Scheme 1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Such a kinetic phase can be reached by having the substrate in great excess compared to enzyme concentration. The following assumptions need to be met during the steady-state to enable mathematical manipulations of the kinetics:

1. The total enzyme concentration is given by the sum of enzyme intermediates and free enzyme.

2. As substrate concentration is much larger than enzyme concentration we can assume that there is no significant reduction of substrate concentration.

3. The rates of intermediate formation and decay are assumed to be constant and equal.

Under these conditions the commonly used Michaelis-Menten expression [163] is deducted for enzyme catalysis rate during steady-state and two important catalytic constants are introduced, k_{cat} and K_M.

$$v = \frac{V_{max}[\mathrm{S}]}{K_M + [\mathrm{S}]} = \frac{k_{cat}[\mathrm{E}]_0[\mathrm{S}]}{K_M + [\mathrm{S}]} \quad (1)$$

What is the value of knowing these constants and what do they really signify? k_{cat} is the number of catalytic turnovers that occur per time unit (normally seconds) and its unit is thus expressed as reciprocal time (s^{-1}). K_M is often mistaken for the dissociation constant of the enzyme substrate complex. This is not the case, K_M merely indicates the substrate concentration at half-maximal rate of the enzyme reaction and approaches K_D for the enzyme substrate complex only when catalysis is slow compared to the off rate of substrate. A better measure of effects on substrate binding and/or chemical steps is the ratio k_{cat}/K_M which is used to define the catalytic efficiency of an enzyme for different substrates or different enzymes for a substrate [164]. k_{cat}/K_M for drug metabolism through the cytochrome P450 pathway is used to predict and model the pharmacokinetics and toxicokinetics of drug candidates.

It is important to bear in mind that even if the steady-state conditions accomplished in an *in vitro* laboratory setting are practical for consistently characterizing and comparing different enzymes, nevertheless, this condition is not typically occurring *in vivo*. *In vivo*, substrate concentrations can be far lower than enzyme concentrations (most relevant to biotransformation enzymes), as has been discussed in the case of GSTs [25]. Furthermore, it is relevant to mention that enzyme catalyzed reactions are rarely as simple as Scheme 1, and often consist of a multitude of steps, where the steady-state velocity will largely reflect the slowest step. In order to more deeply investigate the individual steps of a multistep reaction, transient kinetic methods need to be employed.

2.3.2 Pre Steady-State Kinetics

Steady-state kinetic experiments give substantial biochemical insight into enzymatic reactions. However, there are limitations to this method which make it difficult to derive rate information on the individual binding, chemical or conformational steps on the reaction pathway. To overcome such limitations, rapid kinetic methods are employed to facilitate detection of transient species and will hereon be referred to as pre steady-state kinetics [165].

Pre steady-state kinetics can detect reactions down at a millisecond time scale by means of a specialized apparatus, or even lower by temperature jump techniques [166]. A very commonly employed method is stopped-flow where formation, or decay, of a transient species is detected, usually through an absorbance or fluorescence signal (Figure 2). This pre steady-state technique requires high amounts of enzyme to enable detection of enzyme-substrate/intermediate species.

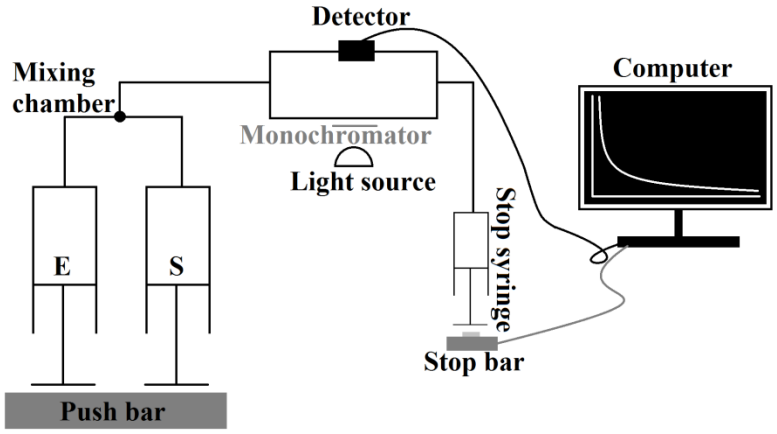


Figure 2. Schematic diagram of a stopped-flow instrument employed for rapid kinetic measurements.

To facilitate the characterization of individual steps of the reaction mechanism the reaction should, whenever possible, be carried out under conditions where the observed kinetics are first order. Usually, this is achieved by making all other concentrations large relative to the concentration of the species of interest. One-step reversible mechanisms give a linear dependence of the observed rate k_{obs}. The observed rate for a one-step binding mechanism will be the sum of the forward and reverse microscopic rates (Scheme 2, Equation 2). A plot of k_{obs} as a function of substrate concentration will give k_{-1} at the intercept and k_1 as the slope of a straight line [167].

Scheme 2.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$k_{obs} = k_1[S] + k_{-1} \tag{2}$$

A more complex case is the investigation of a two-step reversible mechanism (Scheme 3, Equation 3), and mechanisms of interest often consist of multiple steps. This case can be observed if substrate binding is a rapid equilibrium reaction and formation of an intermediate is the rate limiting step. In a plot of k_{obs} as a function of substrate concentration in this case, the maximal observed rate will correspond to the sum of k_2 and k_{-2} and the intercept will equal k_{-2}. Lastly, the initial slope will represent an apparent second order rate constant for substrate binding, $K_1 k_2$, (where K_1 is the association constant and equals $1/K_D$) [168].

Scheme 3.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EX$$

$$k_{obs} = \frac{K_1 k_2[S]}{K_1[S] + 1} + k_{-2} \tag{3}$$

Enzyme mechanism can be far more complex than described here and many researchers have shown proof of ingenuity by applying elegant solutions to detect intricate relations. Today, researchers also have a significant advantage in powerful computers which are able to perform advanced data simulations and fewer simplifications need to be made. By employing specialized data simulation programs for transient enzyme kinetics we can now determine more exact reaction mechanisms when characterizing enzymes [169, 170].

2.3.3 Inhibition

A substantial part of enzyme characterization is focused on investigating inhibition of enzyme-catalyzed reactions. A large part of the enzymes introduced in this thesis are involved in the pathophysiology of chronic, inflammatory diseases including arthritis and cancer, and are thus investigated as potential therapeutic targets both by academia and the pharmaceutical industry. It is important to determine a set of parameters for the evaluation of enzyme inhibitors *in vitro*. Generally, enzyme inhibitors are evaluated through dose-response plots to generate an IC_{50} value, which corresponds to the inhibitor concentration that gives 50% inhibition, referred to as the IC_{50} value that is used to compare relative inhibitor potency. IC_{50} determination is an effective way of studying inhibition for numerous compounds at the same time and is thus a method often employed when screening potential drug candidates in a high throughput setting [171].

It is important to be aware of the limitations for comparing inhibitor potencies by only determining IC_{50} values. The IC_{50} value will depend on a wide variety of conditions:

1. Inhibitor concentrations. It is important to have a large span of concentrations and also have a good precision in the vicinity of the IC_{50} value.

2. Substrate concentration will have a great effect on the IC_{50} value and needs to be kept constant when comparing different inhibitors.

3. Also the relationship between substrate, inhibitor and enzyme concentrations are crucial. It is important to maintain steady-state conditions throughout the assays.

A better way of characterizing inhibitors is to fully determine the specific enzyme inhibition mechanism by which they act. These mechanisms can firstly be divided into two main groups; reversible and irreversible inhibition. In drug discovery the most common mechanism of use is that of reversible inhibition. Reversible inhibition is desired as a feature of a drug for natural preservation of enzymes and receptors and avoidance of adverse effects. Therefore only reversible inhibition will be considered onwards. Enzyme-inhibitor interactions usually function as normal protein-ligand reactions, and the same thermodynamic terms apply for these reactions. In the case of enzyme inhibition, the equilibrium dissociation constant is represented by K_i [171].

Many different types of reversible inhibitors exist, but this discussion will focus on the following: competitive, non-competitive, uncompetitive, mixed and partial inhibitors.

Competitive inhibitors generally bind at the enzyme's active site and consequently compete with the substrate for access to enzyme molecules. This type of inhibition can thus be overcome by sufficiently high substrate concentrations. Non-competitive inhibitors display equal affinity for both the free enzyme and the enzyme-substrate complex. Hence, the extent of inhibition will only depend on the non-competitive inhibitor's concentration. Few commercially available drugs are non-competitive inhibitors. Uncompetitive inhibitors bind exclusively to the enzyme-substrate complex, and show little or no affinity for the free enzyme form. As a result, an increasing substrate concentration cannot overcome the inhibition effect. Mixed inhibitors can be considered as a type of non-competitive inhibitors, where the inhibitor displays finite but unequal affinity for the two enzyme species (free and substrate bound). Partial inhibitors cannot fully block the activity of the enzyme, even at very high concentrations. Partial inhibitors are very rare and it is important to rule out experimental artefacts, such as limited solubility, when encountering this mechanism.

For a competitive inhibitor, the IC_{50} value will depend on the substrate concentration. The IC_{50} value will increase linearly with increasing substrate concentration. For uncompetitive inhibitors the plot of IC_{50} value as a function of substrate concentration will curve downward sharply. For non-competitive inhibitors the IC_{50} will be independent of the substrate concentration, and for mixed inhibitors it will curve upward or downward (Figure 3) [172].

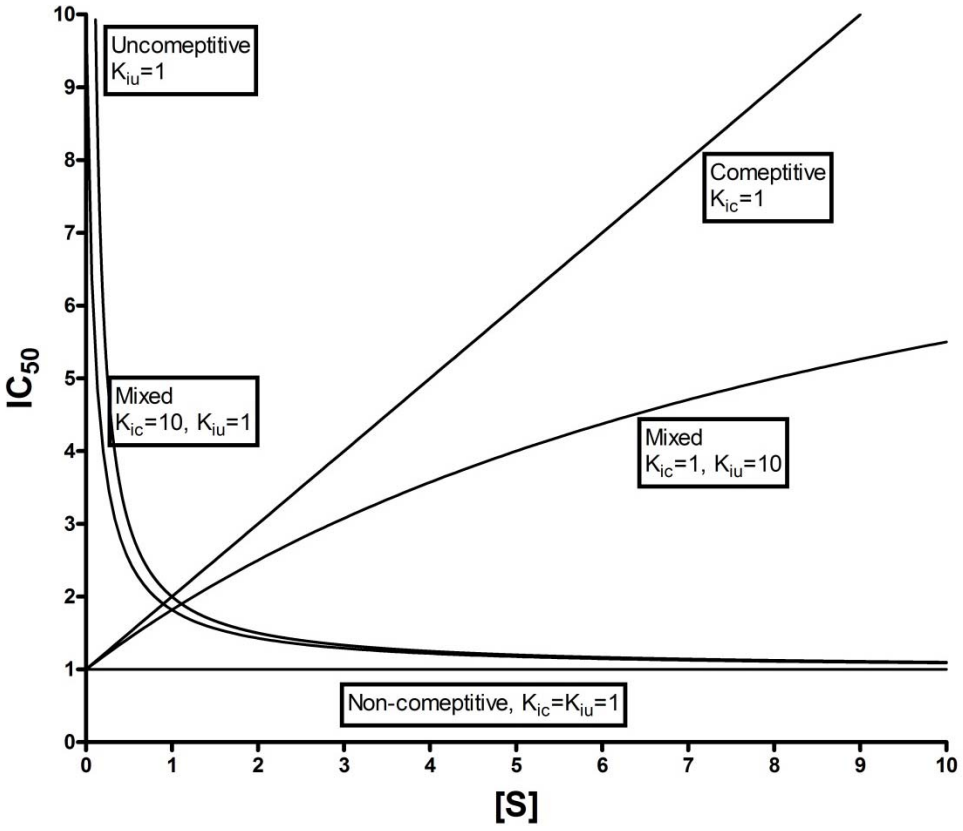


Figure 3. Inhibition mechanism patterns where IC_{50} is plotted as a function of substrate concentration for different inhibition mechanisms (adapted from [173]).

3 METHODOLOGY, CHAPTER 3

In the following section the most relevant methods for the author's contribution throughout papers I-IV will be presented and discussed. This section will primarily serve as a discussion and will not provide detailed practical information. For such details the reader is referred to the methodology section of the articles.

3.1 ACTIVITY ASSAY FOR PGH_2 UTILIZING ENZYMES

An important part of the methodology of this thesis has been based on the development of enzymatic assays for measurements of enzyme activity employing PGH_2 as substrate and has resulted in the publication of a methodological study (paper II). PGH_2 is a very unstable compound and precautions need therefore to be taken when using it as substrate in a biochemical assay. PGH_2 decomposes non-enzymatically in aqueous solution, with a half-life of approximately 5 min at 37°C, into a mixture of PGE_2 and PGD_2 [69, 174, 175]. Accordingly, all MPGES1 activity assays employed in this body of work have been conducted on wet ice to maintain a low temperature of 4˚C. Furthermore, catalysis by enzymes that utilize PGH_2 is normally very fast, substrate depletion easily can occur within seconds, and therefore incubation times need to be short (approximately 1-2 min). Consequently, it has been of great interest for us to develop simple and efficient strategies for activity measurements, where PGH_2 is used as substrate, suitable for a medium-throughput setting.

The principles of the assay employed for PGH_2 converting activity measurements were first reported by Basevich *et al*. [176] and are based on indirect detection of remaining substrate rather than that of direct measurement of product formation. There are several reasons to our choice of method. First, it is a fast method where the degradation product of PGH_2, malondialdehyde (MDA), forms a fluorescent conjugate with thiobarbituric acid (TBA) that can easily be quantified through colorimetric detection. Second, this assay is suitable for handling a large amount of samples and thus issuitable for medium and high-throughput screening, as the reaction can take place in 96 or 384 well plates. A solution of $FeCl_2$ is added in order to entirely stop the reaction after the desired amount of time. $FeCl_2$ non-enzymatically decomposes the remaining PGH_2 into MDA and 12-hydroxyheptadecatrienoic acid (12-HHT) (Figure 4). MDA in turn reacts with TBA forming a red product (a TBA-MDA conjugate) that absorbs at 535 nm [177]. In other words, the amount of formed conjugate in each sample will be inversely proportional to product formation. $FeCl_2$ will not convert other prostaglandins into MDA, this process occurs exclusively for the unstable endoperoxide PGH_2. Of note, other molecules can occasionally form MDA upon reaction with TBA and therefore potential inhibitors are always examined in this respect.

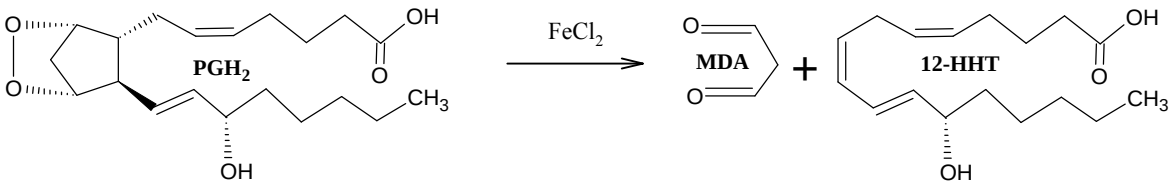


Figure 4. Non-enzymatic decomposition of PGH_2 into malondialdehyde (MDA) and 12-hydroxyheptadecatrienoic acid (HHT) by $FeCl_2$.

MPGES1 catalyzes the isomerization reaction of PGH_2 into PGE_2 [104]. Thorén and Jakobsson described a quantitative assay for MPGES1 activity at the beginning of this century, based upon separation by reverse-phase high-performance liquid chromatography (RP-HPLC), product detection by UV-spectrophotometry and quantification through an internal standard [105]. Advantages of employing this assay include direct detection of product formation, the possibility to detect and quantify all reaction metabolites and its high sensitivity, which enables product detection in the picomole range. In this way, low enzyme activity as well as altered product profile (e.g. when analyzing enzyme mutants) can be detected. On the other hand, this method for direct measurement of prostaglandins requires expensive equipment, is time demanding and can clearly not be used in a medium- or high-throughput setting. In comparison, the MDA-TBA assay is more robust, faster and can process a much larger amount of samples. Even though the colorimetric assay requires high enzyme activity [178] and doesn't provide a full product profile, for screening of numerous MPGES1 inhibitors, the MDA-TBA assay format is the method of choice. The IC_{50} values obtained with this assay can vary 2-3 fold between tests, but that is comprehensible, as this is not a very exact assay (common for assays based on substrate depletion). This is compensated by the fact that it can easily be repeated and more exact IC_{50} values determined by reproduction.

In order to find inhibitors that are truly specific for MPGES1, we have further developed the MDA-TBA assay format to counter-screen our inhibitors in other PGH_2 utilizing enzymes. Those enzymes include MPGES2, PGIS, LPGDS and HPGDS. MPGES2 and LPGDS were quite challenging to assay with the MDA-TBA assay, as they lacked potent reference inhibitors. Even if MPGES2 has been co-crystallized with imidazole [179], we could not detect any inhibition by this compound in MPGES2 (data not shown). In the case of LPGDS, a reference inhibitor has been reported [180], but it was not potent enough to give a full IC_{50} curve in our assay. Low enzyme activity was also an issue. As pointed out above, the MDA-TBA assay format requires high activity enzyme and that was not the case with Cayman Chemicals LPGDS enzyme preparations. Eventually, we proceeded to apply an enzyme immunoassay (EIA) for LPGDS. The reference inhibitors used for PGIS and HPGDS were U-51605 [181] and HQL-79 [182] respectively. As this assay detects only consumed substrate, it is difficult to determine whether a partial inhibitor is truly partial or if it is due to artefacts. Therefore, reference inhibitors are an important part of the MDA-TBA assay, as they can demonstrate full inhibition.

3.2 INHIBITOR STUDIES

There is a great difference in approach when testing inhibitors in a high-throughput setting compared to mechanistic and detailed studies of a few inhibitors of interest. In the high-throughput setting one needs to have flexible methods, which can detect a wide range of potency, be able to process a large number of samples, thus being rather uncomplicated (preferably automatized) and economic in terms of cost and time spent on each tested sample. Robustness and consistency are two additional features which are very important, as the results are compared over a time period that could be several years long. This process might sound tedious for the free spirit of many researchers, but its rewards are often potent inhibitors that can bring much excitement as they can be applied in a wide array of systems thanks to their strong effect. At this point, more expensive and time demanding methods can be employed and potent inhibitors can develop into tools for the study of enzyme inhibition [183].

The workflow when developing drug candidates for MPGES1 inhibition started by screening tens of thousands of compounds from commercially available libraries in the colorimetric assay. MK-886 was used as a reference inhibitor based on the large number of publications where its IC_{50} in MPGES1 has been reported [184-189]. MK-886 was initially tested in MPGES1 because of its potent inhibitory effects on two other members of the MAPEG family: LTC_4S and FLAP [184]. The A549 non-small cell lung cancer cell line was selected for similar reasons when evaluating inhibitors in our *in vitro* cellular assays. A459 cells have been reported to have an increased COX2 and MPGES1 expression after IL-1β stimulation [190] and have been used in cellular assays to evaluate novel MPGES1 inhibitors [123, 191]. After evaluating inhibitor potency *in vitro*, the most potent hits were further optimized and their specificity was evaluated in parallel. Counter-screening assays included the colorimetric MDA-TBA assay for MPGES2, PGIS, LPGDS and HPGDS, as well as COX1 and COX2 EIAs. The most promising compounds were further evaluated for species selectivity (paper I), inhibition mechanism (paper II) and finally in animal models (paper III).

3.3 STOPPED FLOW EXPERIMENTS

In the introduction, the theoretical aspects of transient kinetics were presented. In order to be able to determine the microscopic rate constants, a measurable signal must exist for one or preferably several of the reacting species/intermediates. It is also necessary that those species change their spectroscopic properties during the course of the reaction. Luckily, there are several measurable signals that can be detected in the reaction pathway of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with MGST1 [192] and these were applied in paper IV.

To be able to conduct the stopped-flow experiments at 5˚C, the stopped-flow apparatus (including the syringes, mixing chamber and detection cell) is equipped with a water cooler (or heater if required). In this manner, the reaction temperature could be monitored and

constantly kept at 5˚C. In a stopped-flow apparatus, the path of the cell where the absorbance signal is detected is normally 10 mm long, whereas the fluorescence signal is detected in a 2 mm path length configuration.

The absorbance signal for thiolate anion formation in MGST1 can be followed at 239 nm (ϵ_{239} = 5000 $M^{-1}cm^{-1}$), and is considerably higher than that of GSH at 239 nm. At high GSH concentrations on the other hand, the GSH background signal becomes so high that we had to employ a 2 mm cell path. A shorter path length will give a lower signal. CDNB also has an absorbance at 239 nm (ϵ_{239} = 2700 $M^{-1}cm^{-1}$), which is higher than that for the GS-CDNB conjugate.

When performing single turnover stopped-flow experiments GSH containing MGST1 was injected from one syringe and its second substrate, CDNB, from the other syringe. MGST1 displays one-third-of-the-sites reactivity [193] and will already have converted one of the GSH molecules in its active sites into GS^-. When the two solutions are rapidly mixed all active sites containing GS^- molecules will quickly conjugate a CDNB molecule (burst) and both the thiolate anion and CDNB signal will simultaneously decrease. The amplitude of the observed signal is actually an active site titration. This amplitude can thus account for factors such as inactive enzyme. The reason we can detect this signal is that there is a rate limiting step after the conjugation reaction, which consists of slow thiolate anion formation after a fast GSH rebinding step [194]. It is rather unusual that the thiolate anion formation is the rate limiting step in a GST mechanism, although examples have been reported [195], as it is normally a fast step compared to turnover [195-198]. As microscopic steps involving deprotonation are typically very fast, we believe that the slow step rather reflects a conformational transition that limits deprotonation [194]. Activation of MGST1 by sulfhydryl reagents actually increases the rate of thiolate anion formation [199]. Depending on the reactivity of the second substrate, either this microscopic rate or chemical conjugation can be rate limiting for the overall turnover [200].

If there is an excess of CDNB, the experiment no longer is a single turnover experiment, but rather a limited turnover experiment (Figure 5). In this case, the remaining CDNB will be consumed and the corresponding turnover rate will depend on the rate limiting step of the overall catalytic mechanism (thiolate anion formation). When all the CDNB is consumed we can follow this slow step of the enzyme mechanism separately as the enzyme-thiolate rebound. Compared to the burst, the amplitude of the signal here will be lower, as it only involves reformation of the GSH thiolate. Thus limited turnover experiments constitute a powerful approach to measuring the active site concentration, chemical rate, turnover rate and thiolate anion formation rate in a single experiment. Importantly, the experimental conditions reflect the GSH bound enzyme as it functions *in vivo*.

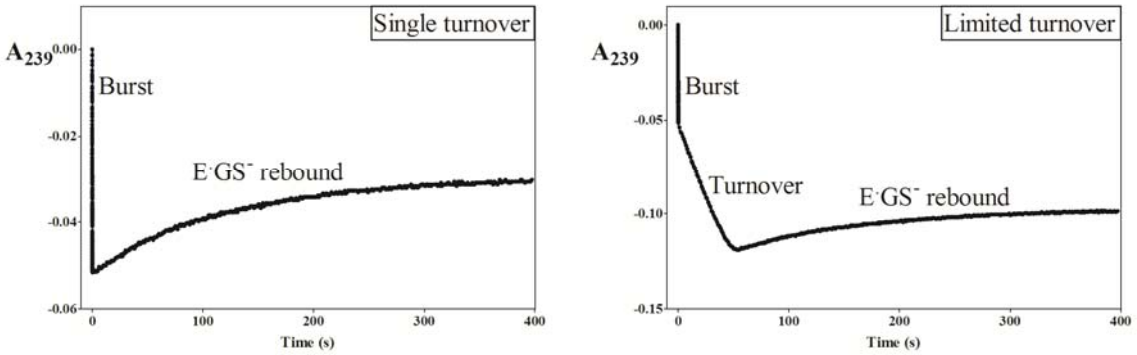
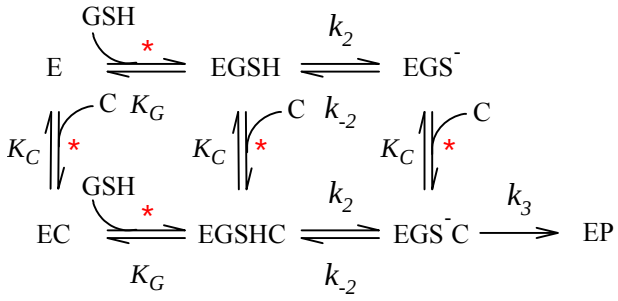


Figure 5. Example of a single turnover trace compared to a limited turnover trace for the reaction that takes place when GSH containing MGST1 is rapidly mixed with CDNB in a stopped flow experiment. Absorbance was measured at 239 nm.

3.4 DERIVATION OF THE STEADY-STATE RATE EQUATION FOR MGST1

Derivation of the steady-state rate equation for enzyme mechanisms can be achieved by following the three assumptions listed in the introduction section on steady-state kinetics (total enzyme concentration is equal to the sum of free enzyme and intermediate species, no change in substrate concentration, and finally, rates of formation and decay of intermediates are assumed to be constant and equal). First, a set of equations for mass balance and all the involved reactions and equilibria (of the different enzyme forms) are set up, and subsequently the rate equation is derived by solving the postulated equations. This is how we derived the steady-state rate equation for MGST1. Simplifying assumptions in the particular case of MGST1 are equal binding affinity of substrates to the various enzyme forms and treatment of the re-protonation rate process using an apparent first order constant. As complex enzyme mechanisms can result in intricate equations, several methods have been developed to simplify the derivation process. One is the traditional King Altman method from 1956 [201], which has been further developed for reactions where equilibria are involved by i.e. Cha *et al*. [202], an approach that we also have employed to confirm our resulting rate equation (not shown). Here we show the complete derivation of the steady-state rate equation for MGST1 based on the random sequential bisubstrate mechanism shown in Scheme 5 where C represents an electrophilic substrate and rapid equilibria are denoted with an asterisk.

Scheme 5.



Total enzyme concentration is equal to the sum of free enzyme and intermediate species:

$$[E_t] = [\mathrm{EC}] + [\mathrm{EGSH}] + [\mathrm{EGSHC}] + [\mathrm{EGS^-}] + [\mathrm{EGS^-C}] + [\mathrm{E_f}]$$

Equilibrium relations based on the mechanism (Scheme 5) give expressions for the different enzyme species based on the equilibrium binding constants for GSH and the electrophilic substrate (C):

$$K_C = \frac{[\mathrm{EGS^-}][\mathrm{C}]}{[\mathrm{EGS^-C}]} \quad \Rightarrow \quad [\mathrm{EGS^-}] = [\mathrm{EGS^-C}]\frac{K_C}{[\mathrm{C}]}$$

$$K_C = \frac{[\mathrm{EGSH}][\mathrm{C}]}{[\mathrm{EGSHC}]} \quad \Rightarrow \quad [\mathrm{EGSHC}] = [\mathrm{EGSH}]\frac{[\mathrm{C}]}{K_C}$$

$$K_G = \frac{[\mathrm{EC}][\mathrm{GSH}]}{[\mathrm{EGSHC}]} \quad \Rightarrow \quad [\mathrm{EC}] = \frac{K_G[\mathrm{EGSHC}]}{[\mathrm{GSH}]} = [\mathrm{EGSH}]\frac{K_G[\mathrm{C}]}{K_C[\mathrm{GSH}]}$$

Insertion of the above relations into the formula for K_G give us expressions for [EGSH] and [EGSHC] including [$\mathrm{EGS^-}$] and [$\mathrm{EGS^-C}$]:

$$K_G = \frac{[\mathrm{E_f}][\mathrm{GSH}]}{[\mathrm{EGSH}]} = \frac{([\mathrm{E_t}] - [\mathrm{EC}] - [\mathrm{EGSH}] - [\mathrm{EGSHC}] - [\mathrm{EGS^-}] - [\mathrm{EGS^-C}])[\mathrm{GSH}]}{[\mathrm{EGSH}]}$$

$$\Rightarrow \quad \frac{K_G}{[\mathrm{GSH}]} = \frac{[\mathrm{E_t}] - [\mathrm{EGS^-}] - [\mathrm{EGS^-C}]}{[\mathrm{EGSH}]} - \frac{K_G[\mathrm{C}]}{K_C[\mathrm{GSH}]} - \frac{[\mathrm{C}]}{K_C} - 1$$

$$\Rightarrow \quad [\mathrm{EGSH}] = \frac{[\mathrm{E_t}] - [\mathrm{EGS^-}] - [\mathrm{EGS^-C}]}{\frac{K_G[\mathrm{C}]}{K_C[\mathrm{GSH}]} + \frac{K_G}{[\mathrm{GSH}]} + \frac{[\mathrm{C}]}{K_C} + 1}$$

$$\Rightarrow \quad [\mathrm{EGSHC}] = \frac{[\mathrm{E_t}] - [\mathrm{EGS^-}] - [\mathrm{EGS^-C}]}{\frac{K_G K_C}{[\mathrm{C}][\mathrm{GSH}]} + \frac{K_G}{[\mathrm{GSH}]} + \frac{K_C}{[\mathrm{C}]} + 1}$$

Equal rates of formation and decay of the intermediates give the following equation:

$$k_2([\mathrm{EGSH}] + [\mathrm{EGSHC}]) = k_{-2}([\mathrm{EGS^-}] + [\mathrm{EGS^-C}]) + k_3[\mathrm{EGS^-C}]$$

By substituting the above derived expressions for [EGSH], [EGSHC] and [$\mathrm{EGS^-}$] into the following equation we have all we need to derive the steady-state rate equation:

$$k_2\frac{[\mathrm{E_t}]-[\mathrm{EGS^-}]-[\mathrm{EGS^-C}]}{\frac{K_G[\mathrm{C}]}{K_C[\mathrm{GSH}]}+\frac{K_G}{[\mathrm{GSH}]}+\frac{[\mathrm{C}]}{K_C}+1}+k_2\frac{[\mathrm{E_t}]-[\mathrm{EGS^-}]-[\mathrm{EGS^-C}]}{\frac{K_GK_C}{[\mathrm{C}][\mathrm{GSH}]}+\frac{K_G}{[\mathrm{GSH}]}+\frac{K_C}{[\mathrm{C}]}+1}$$
$$=k_{-2}[\mathrm{EGS^-}]+k_{-2}[\mathrm{EGS^-C}]+k_3[\mathrm{EGS^-C}]$$

After simplifications, including insertion of the expression for turnover rate, which is determined by the last step of the mechanism (Scheme 5),

$$v=k_3[\mathrm{EGS^-C}]$$

we arrive at the steady-state rate equation for MGST1:

$$v=\frac{k_2k_3[\mathrm{C}][\mathrm{GSH}][\mathrm{E_t}]}{k_{-2}\mathrm{X}+k_3\mathrm{Y}+k_2\mathrm{Z}}$$

$$\mathrm{X}=K_C[\mathrm{GSH}]+K_CK_G+[\mathrm{C}][\mathrm{GSH}]+K_G[\mathrm{C}]$$

$$\mathrm{Y}=[\mathrm{C}][\mathrm{GSH}]+K_G[\mathrm{C}]$$

$$\mathrm{Z}=[\mathrm{C}][\mathrm{GSH}]+K_C[\mathrm{C}]$$

4 THESIS AIMS, CHAPTER 4

The work presented in this thesis is focused on extending the mechanistic understanding of MAPEG superfamily members MPGES1 and MGST1. The overall aim of this thesis project is to elucidate the catalytic and inhibitory mechanisms of these two integral membrane proteins. The specific aims of this study form the basis of the following publications:

Paper I — To investigate the active site and inhibitor binding site of MPGES1 and determine species differences of the human and rat enzyme

Paper II — To evaluate inhibition mode for MPGES1 specific inhibitors

Paper III — To characterize a new pharmacological inhibitor of MPGES1 in murine models of inflammation and compare its effect with MPGES1 gene deletion *in vivo*

Paper IV — To determine the global kinetic mechanism of MGST1 by pre steady-state and steady-state kinetics

Our studies thus potentially contribute to human health by enabling safer drug development and to the basic understanding of membrane bound enzymes.

5 RESULTS AND DISCUSSION, CHAPTER 5

This thesis includes three studies regarding function and inhibition of MPGES1, which have all been published. These publications can be found at the end of this thesis as papers I-III. Paper I deals with structural aspects of MPGES1, paper II is a methodological paper for the evaluation of MPGES1 inhibition *in vitro*, and finally in paper III MPGES1 inhibition is evaluated *in vivo.* The final study, included as paper IV, focuses on MGST1 and is available in the form of a manuscript as it has not yet been published. Paper IV presents the global kinetic mechanism of MGST1. The main findings will be presented and discussed here in the context of relevant literature.

5.1 MPGES1 INHIBITION

MPGES1 has been called a novel therapeutic target since 1999 [104], and continues to be attributed that title [100]. Unfortunately, 15 years later, there are no MPGES1 inhibitors in advanced clinical trials and many pharmaceutical companies have discontinued their R&D efforts on this pharmacological target. This is the reality of many pharmaceutical projects and several take far longer to finally develop into commercially available drugs. This was for instance the case for omeprazole (Losec), where discovery efforts by Astra and Swedish researchers at Sahlgrenska Hospital started already in the 1950's and launch of the drug was in 1988 [203].

Potent inhibitors targeting MPGES1 have been developed, but when testing them in animal models, they often turn out to be quite ineffective. Merck developed a potent and selective MPGES1 inhibitor, MF63, with a reported IC_{50} of 1 nM in enzymatic assays of human MPGES1. This inhibitor however was virtually inactive against rat MPGES1 [123, 131]. We investigated MPGES1 as a drug target in collaboration with Actar and NovaSAID, and during the screening process we found two potent human MPGES1 inhibitors originating from two different screening efforts (Figure 6). Unfortunately, we encountered the same problems as Merck did with several of our hits when attempting to characterize them with rat enzyme. Compound I only inhibited human MPGES1, whereas compound II inhibited both enzyme orthologues. To investigate the underlying details of this discrepancy in species specific inhibition, we evaluated the enzyme and inhibitor differences through site directed mutagenesis (paper I).

Initially, two chimeric forms of rat/human MPGES1 were created to determine the approximate range within which the catalytically active and inhibitor interacting amino acids could be encountered. Sequence differences between the enzymes of the two species are located in transmembrane helices 3 and 4, as well as the N terminus. We also had guidance from the then recently published electron crystallography structure of MPGES1 [204] and chose to exchange residues 115-140 from the rat enzyme into the human enzyme, and vice versa. The exchanged sections comprise the end of transmembrane helix 3, the beginning

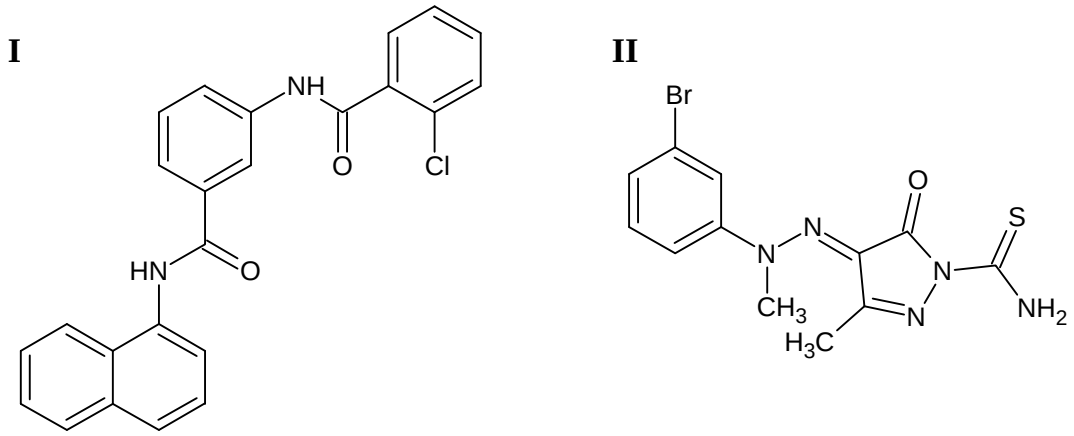


Figure 6. Chemical structures for compounds I and II which are competitive inhibitors of MPGES1 evaluated in papers I and II. 2-Chloro-N-[3-(naphthalen-1-ylcarbamoyl)phenyl]benzamide (I), 4-[2-(3-Bromophenyl)hydrazono]-3-methyl-5-oxo-4,5-dihydro-1H-pyrazole-1-carbothioamide (II).

of transmembrane helix 4 and the cytosolic loop in between them. Both chimeric proteins had PGE_2 forming activity in the same range as the rat enzyme, which is 3 times lower than the human enzyme. When investigating inhibition, we observed that compound I failed to inhibit the catalytic activity of hum115rat140hum and gained inhibitory potential in the rat115hum140rat chimeric enzyme. We could thereby conclude that residues of importance for inhibitor binding were in fact within this chosen region. Subsequently, those amino acids that varied in between the two species were mutated through site directed mutagenesis and investigated for activity and inhibition by compound I. No single mutation accounted for the full effect of inhibitor sensitivity, but a few were more prominent than others, so we continued our efforts and created new mutant combinations based on two or more mutations. The result of our efforts identified mutations V131T, F135L and F138A in the rat enzyme as the most critical for regained inhibitor sensitivity. The IC_{50} for compound I in the rat enzyme triple-mutant still was somewhat higher than that in the chimeric rat protein and wild type human enzyme, which implies that other more remote residues could also contribute to the inhibition sensitivity, although with only less pronounced effects. The three crucial residues are all aligned in the cleft between helix 4 and 1, which is the presumed PGH_2 substrate-binding site (and inferred competitive inhibitor binding site). In fact a recent MPGES1 structure obtained from in cubo crystallization with bound inhibitor is consistent with the location [205]. In human MPGES1, these residues are rather small, but in the rat enzyme they are instead bulky aromatic residues and thereby mediate steric hindrance for several MPGES1 inhibitors. Similar bulky residues are found in the mouse orthologue, whereas they are not encountered in the guinea pig enzyme. This could be the explanation why many human MPGES1 inhibitors have similar IC_{50} values with the guinea pig enzyme [123] and also motivated the use of this species as an animal model. At the time our studies were the first experimental data supporting the PGH_2 and inhibitor binding site and as such could be used for targeted docking efforts. Subsequent enzyme inhibitor structures [143, 205] are of course more conclusive.

In parallel with the above discussed investigations, we determined the mode of inhibition of several inhibitors (including compound I and II) to better understand their inhibitory mechanism. We used the MDA-TBA assay format and determined IC_{50} values for interesting inhibitors which had already been well characterized for potency and selectivity in several *in vitro* assays. The assay was conducted at three different PGH_2 concentrations, one at the K_M value (170 μM) [206], one well below its K_M value (10 μM), and one high above (640 μM). By applying the principles shown in Figure 3, it is possible to determine the inhibition mechanism by investigating the pattern of IC_{50} values as a function of substrate concentration. It is imperative that the substrate concentrations are spread out around the K_M value for the trends to be meaningful. Paper II describes a simple and generally applicable approach to determine inhibition mechanism and inhibitor potency of MPGES1 inhibitors. We felt that there was a need to establish a method for evaluating MPGES1 activity which didn't require demanding procedures [105] or expensive robotics [130]. No previous study had determined the inhibition mechanism of K_i value for the often employed reference inhibitor MK-886. Here we concluded that MK-886 along with compound II showed mixed inhibition towards PGH_2. The remaining four compounds were pure competitive inhibitors towards PGH_2 and all inhibitors were non-competitive towards GSH. Most reported MPGES1 inhibitors are competitive toward PGH_2, as shown here, and also by others [207]. Moreover, as observed in paper I, the residues responsible for inhibitor efficiency are aligned on the cleft where the substrate potentially enters. We suggest therefore that inhibitor binding likely occurs in the same cleft. Prage *et al.* reported their H/D exchange experiments recently after our publication [150], and could confirm that the location of MK-886 include the GSH binding site and the substrate binding cleft. Shortly thereafter, He and Lai published a molecular docking study which supported the idea that MK-886 binds to the PGH_2 binding site [208]. To summarize, we have developed a simple and efficient approach for pharmaceutical inhibitor screening, determined the inhibitory mechanism of the well-established MPGES1 reference inhibitor MK-886, and developed several potent competitive inhibitors of MPGES1.

The promising results for compound II as a rat enzyme inhibitor pushed us to further characterize this compound *in vitro* and *in vivo* [183]. Through this compound we could set up models to test specific MPGES1 inhibitors for their potential in the treatment of inflammation related to arthritis. Those models consisted of the air pouch and adjuvant-induced arthritis models which are driven by the inducible PGE_2 pathway [185, 209] and have been applied in the development of NSAIDs [209, 210]. Our discovery efforts complete this story of MPGES1 inhibition with the development of compound III (Figure 7). It is unfortunate, but important, to note that compound III in paper III differs from compound III in paper II. Hereon, compound III will refer to that described in paper III and Figure 7.

Compound III was developed in a targeted effort to improve the potency for human MPGES1 (IC_{50} of 0.09 μM), whereas its potency in the rat enzyme is in the same range as that of

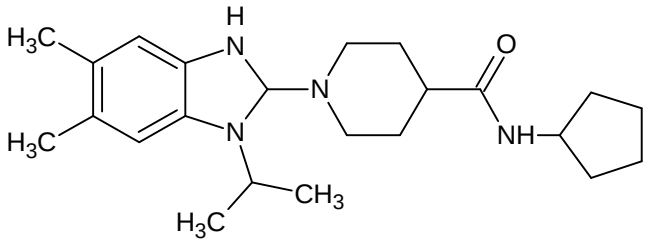


Figure 7. Chemical structure for compound III; 1-(1-isopropyl-5,6-dimethyl-1H-benzoimidazol-2-yl)-piperidine-4-carboxylic acid cyclopentylamide.

compound II (IC_{50} of 0.9 μM) MPGES1. This compound was characterized *in vitro* by the aforementioned enzymatic and cellular assays. We also tested the compound *in vitro* in the presence of serum proteins, as protein binding has been reported to be a potential problem in the development of MPGES1 specific inhibitors [189]. The hydrophobic nature of PGH_2 and therefore its binding site, results in development of MPGES1 competitive inhibitors that are quite hydrophobic. Consequently, they often perform poorly in whole blood assays or assays with high serum protein content [131, 138, 211, 212]. The whole blood assay used in paper III originates from Brideau *et al* [213] and was established for the development of NSAIDs. This assay is perceived as a robust method to assure that inhibitors are likely to function *in vivo*.

In order to evaluate if MPGES1 is a better drug target than COX inhibition by traditional NSAIDs, much effort has been put in assessing the effect of MPGES1 inhibition on the prostanoid profile. Long-term treatment with COXibs has been reported to cause cardiovascular complications associated with perturbation in the blood TXB_2/PGI_2 ratio [214] and it is thus necessary to prove that this is not the case with MPGES1 inhibitors. Treatment with MPGES1 inhibitors and MPGES1 gene deletion have been reported and point to a heterogeneity in outcomes, which are dependent on cell type, assay conditions and genetic background [120, 123, 129, 138, 211, 215]. This is in line with our results, where MPGES1 inhibition by compound III in A549 cells was associated with shunting of PGH_2 into the prostacyclin pathway, whilst it induced general down-regulation of prostanoid synthesis in the mouse air pouch model. Moreover, genetic deletion of MPGES1 in the mouse air pouch model and mouse macrophages shunted PGH_2 into the thromboxane pathway. Differences in the effect of MPGES1 pharmacological inhibition and genetic deletion are to be expected, as inhibition is never complete and doesn't directly affect enzyme expression. It is therefore important to compare inhibitors in the same models with similar conditions and to complement results from knockout animals with inhibition results in wild type systems.

Through different *in vitro* assays and *in vivo* models of inflammation we have characterized compounds II and III. At the time these compounds were the only MPGES1 inhibitors reported to have an effect *in vivo* in native murine models of inflammation. Our results show that it is possible to develop potent human MPGES1 inhibitors that can be tested in murine *in vivo* models.

Where do MPGES1inhibition efforts stand now? Except for compound III, which is a potent inhibitor that has a demonstrated effect in animal models, the study by Shiro *et al*. of new, potent MPGES1 inhibitors appears promising [216]. Their inhibitors seem to have a strong profile including nanomolar range IC_{50} values in enzymatic and cell-based assays, good MPGES1 selectivity, and favorable *in vitro* ADME and *in vivo* rat PK profiles. New approaches in inhibitor development will be discussed in the future perspectives section.

5.2 THE GLOBAL MECHANISM OF MGST1

MGST1 is the closest relative to MPGES1 in the MAPEG superfamily based on sequence similarity (38%) [147]. MGST1 has been far more studied, especially concerning its enzymatic mechanism, than MPGES1, and even if there are clear differences in their biological functions and catalytic mechanisms, many important aspects of one's catalysis can give insights to the other's.

Our group was involved in the discovery and characterization of MGST1 [30-34] and has further investigated the details of its catalytic mechanism in detail [30, 37, 193, 194, 199, 217]. As was described in the methodology section, MGST1 displays a bimolecular random sequential mechanism [37] and the ability to be activated, up to 30-fold, by modification with sulfhydryl reagents [30, 31]. Moreover, MGST1 exhibits one-third-of-the-sites-reactivity towards GSH [193] and hence heterogeneous binding at its three active sites. Pre steady-state kinetic steps have been characterized for MGST1 [193, 194, 199], but until now, global mechanisms have only been determined for soluble GSTs [198, 218-220]. In paper IV, we assemble the collected knowledge on the different steps of the catalytic mechanism of MGST1 and propose a global mechanism for this enzyme. Through limited turnover kinetic measurements of the activated enzyme form, we could more accurately determine K_D for the "third" low affinity GSH-binding site (1.4 ± 0.3 mM) and the rate of thiolate formation, k_2 (0.77 ± 0.06 s^{-1}). These constants are more physiologically relevant compared to the high K_D values for GSH obtained earlier [199]. Intracellular GSH concentrations will thus saturate MGST1 *in vivo* and ensure that the enzyme is fully loaded with GSH at the start of a catalytic cycle [25]. A Hammett analysis was also conducted on four second substrates of increasing chemical reactivity. The results differ from those obtained at 30˚C [200]. In general, the kinetic parameters at 5˚C are linearly dependent on the electrophile's reactivity at the same time as activation of the enzyme clearly takes place regardless of chemical reactivity of the second substrate. MGST1 seems to enter a resting state at these low temperatures, which can be activated both in terms of increased rate of thiolate formation and in the chemical conjugation step. By deriving the steady-state rate equation we compared the theoretical catalytic constants K_M, k_{cat} and k_{cat}/K_M (obtained by inserting the microscopic constants in the equation) to the experimentally obtained constants. The microscopic steps account for the global mechanism in activated enzyme with reactive second substrates. The catalytic constants obtained for reactive substrates in unactivated MGST1 can only be accounted for by the microscopic constants if a more active subpopulation of MGST1 is assumed. The

existence of an activated subpopulation (approximately 10%) could be directly demonstrated in limited turnover experiments. In conclusion, we suggest that MSGT1 has a pre-existing dynamic equilibrium between high and low activity forms.

The activated MGST1 is unstable without GSH at 30˚C and pre steady-state analysis therefore had to be performed at 5˚C. Our conclusions are thus valid for the enzyme's catalytic behavior at low temperature. Still, there are important lessons to be learned from this investigation. Furthermore we propose limited turnover experiments, which can be conducted at 30˚C, to obtain physiologically relevant results. These ideas will be further discussed in the future perspectives.

5.3 FUTURE PERSPECTIVES

5.3.1 Global mechanism of MGST1

Hammett analysis and steady-state experiments of MGST1 with a series of four electrophilic second substrates at 5˚C pointed to a dynamic resting state of this enzyme (paper IV). As this phenomenon was not observed at 30˚C, we propose that the resting state is more peculiar to the low temperature experiments.

By performing limited turnover stopped flow experiments at 30˚C we will obtain physiologically relevant microscopic rate and equilibrium constants. By insertion of these constants in the steady-state rate equation derived here, we will be able to evaluate if the pre steady-state behavior can account for the published steady-state kinetic behavior at 30˚C. These experiments are feasible, since the enzyme is stable in the presence of GSH at this temperature and would give a good basis for the understanding of the catalytic mechanisms of other MAPEG family members.

5.3.2 Inhibition of MPGES1 targeting the GSH binding site

Hurdles in the development of PGH_2 competitive inhibitors of MPGES1 include their species specificity, hydrophobic nature and solubility issues. We propose investigation and development of inhibitors targeting the enzyme's GSH site instead. By targeting the GSH site, enhanced solubility could more easily be achieved for potential inhibitors. GSH is a hydrophilic molecule which enters the active site of MPGES1 from the cytosol rather than through the phospholipids of the membrane, which is the route of entrance for PGH_2. In this way, problems encountered with limited availability of animal models, binding to serum proteins and bioavailability could be diminished.

He *et al*. have in their study from 2011 explored the idea of development of dual-site inhibitors [208], but, to our knowledge, no groups have explored the possibility of developing GSH competitive and PGH_2 non-competitive inhibitors in MPGES1. In LTC_4S, this notion has been fully explored by Ago *et al*. who found a series of GSH competitive inhibitors which were non-competitive towards LTA_4 [221]. Prague *et al*. have explored the binding of

glutathione sulfonate in MPGES1 through H/D exchange experiments [150]. They also found that this inhibitor has a rather high IC_{50} (1.8 mM) in MPGES1 compared to other GSH utilizing enzymes [222]. Several groups have also made efforts to develop and characterize dual inhibitors of MPGES1 and 5-LO for the inhibition of pro-inflammatory PGE_2 and leukotrienes formation [223-227]. Sjögren *et al.* from AstraZeneca confirmed the horse-shoe shaped conformation of GSH in the active site of MPGES1 [143] for which there was evidence already in the crystal structure determined by our groups through electron crystallography [204]. This conformation of GSH is different to that of any other investigated GSH utilizing enzyme (except LTC_4S [228]) and could therefore be an interesting way of proceeding with drug development efforts. Dual inhibitors of MPGES1 and LTC_4S could be pursued as a potential pharmacological strategy for treatment of inflammation and cancer, and a more specific alternative to dual inhibitors of MPGES1 and 5-LO.

5.3.3 Catalytic mechanism for MPGES1

MPGES1 has been extensively investigated as a pharmaceutical target and great efforts have been made in developing inhibitors targeting its active site, though very little is known about its catalytic mechanism. Two catalytic mechanisms have been proposed on the basis of the electron crystallography [204] and X-ray structures [143] but remain to be confirmed. As the latter has a higher resolution and more detail, we propose to explore that mechanism based on site directed mutagenesis and pre steady-state experiments. The catalytically interesting residues to mutate in human MPGES1 would be those close to, or coordinating the GSH molecule (Ser127, Arg126, Asp49 and Arg73).

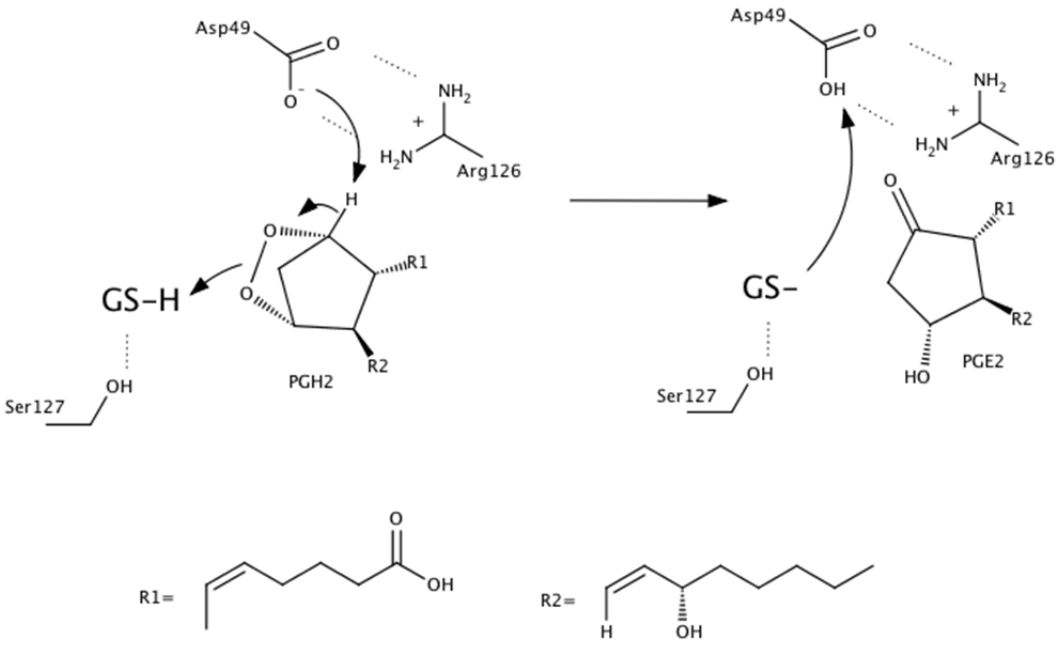


Figure 8. Proposed catalytic mechanism for MPGES1 where the catalytic cycle is initiated by proton abstraction at C9 (by Asp-49) and the protonated GSH thiol acting as a proton donor to the developing oxyanion at C11 in PGH_2 perhaps assisted by Ser-127. Subsequent proton reshuffling from Asp-49 to GSH thiolate forming the catalytically competent ground state could involve water molecules present in the active site.

Preliminary results from our mutagenesis studies show that Arg126 and Asp49 are important residues for the enzymes catalytic activity, whereas the Ser127 and Arg73 mutants retain almost full activity (data not shown). Our results are preliminary and were evaluated through Western Blot for MPGES1 expression in bacterial membrane fractions, MDA-TBA assay for activity and RP-HPLC coupled to a mass spectrometer (RP-HPLC/MS) for product analysis. Based on these findings and difficulties in detecting a thiolate signal in stopped flow experiments (performed in collaboration with Haeggström *et al*.) we propose a novel catalytic mechanism for MPGES1 (Figure 8).

MPGES1 is a less stable protein than MGST1; it precipitates without GSH and has a very labile substrate. All these aspects make enzymatic and mechanistic characterizations of this protein more complicated. Still, understanding the catalytic mechanism of MPGES1 will play an important role in the continued understanding of the MAPEG superfamily of enzymes, as well as in the development of future inhibitors and potential new drugs.

6 ACKNOWLEDGEMENTS

First and foremost I would like to thank my main supervisor Ralf for being an amazing teacher and giving me the wonderful opportunity to collaborate in your projects. You have been a great source of inspiration, even if you don't like to high-five at great results. The open-minded, positive and tolerating atmosphere in your group has made me able to conquer many obstacles. I will always strive to be as optimistic as you are and would gladly collaborate with you on many more scientific projects in the future.

I have been very lucky to have a fantastic network of co-supervisors throughout my time in the "MAPEG-family". Per-Johan, thank you for being so welcoming and making me feel as a member of your group. I have truly enjoyed our discussions and appreciate your enthusiasm and passion for science. Hans and Richard, I appreciate our discussions and your great interest in my work. Sorry for the short deadlines and thanks for all the support.

Pirkko, having your support during this time has been a wonderful experience. Your guidance has made me grow both on a professional and personal level. Thanks for everything!

Another great group of people with whom I've learned a lot about MPGES1 (and writing reports) are my former colleagues from Actar. Patric and Charlotte, thank you for believing in me and paving the way for this PhD adventure. Sara, Björn, Ulrika, Eva, Natalia, Johan W, Mathias, Delal, Sonja thanks for great times which I will always cherish.

Astrid, thanks for the long talks about everything in-between life and work, I look forward to more very soon. Nazmi, I already miss your happy face in the lab, thanks for all the great work. Joan, Patrick, Sven, Helena, Rao, Karin, Marina, Caroline it has been a wonderful collaboration and exciting time to explore MPGES1 with you! Neus, thanks for being a great friend during this last year; I hope it will continue for many more. Ian J, Åse, Aram, Sandeep, Ilona, Kristin, thank you for all your help in the lab and for great fun at lunch, fika and AW. Ulla, Johan, Ian C, thanks for interesting discussions and great questions at my presentations.

Johan Å, Richard, Carina, thanks for the collaboration concerning MGST1. I would also like to thank all other collaborators with whom I have projects outside of the scope of my thesis; Katarina, Kristian and Lars, thanks for letting me in on a very exciting project. Jesper, Agnes, Michaela, Shabbir, thanks for the opportunity of a new and exciting MPGES1 project.

Henke och Hansi, tack för ert engagemang och all hjälp runt min disputationsfest, ni är otroliga! Anna, Malin, Sigrid, Therese och Viktorija tack för att ni alltid finns vid min sida och lyssnar på mina bekymmer och glädjeämnen. Utan alla er skulle det inte ha varit en lika rolig resa!

Mbi të tërë, dua të falenderoj familjen time të dashur. Mami, babi, Eriana, John, nuk e di si do t'ia kisha dalë dot pa ndihmën e vazhdueshme tuaj. Shumë, shumë faleminderit, ju kam shumë xhan!

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