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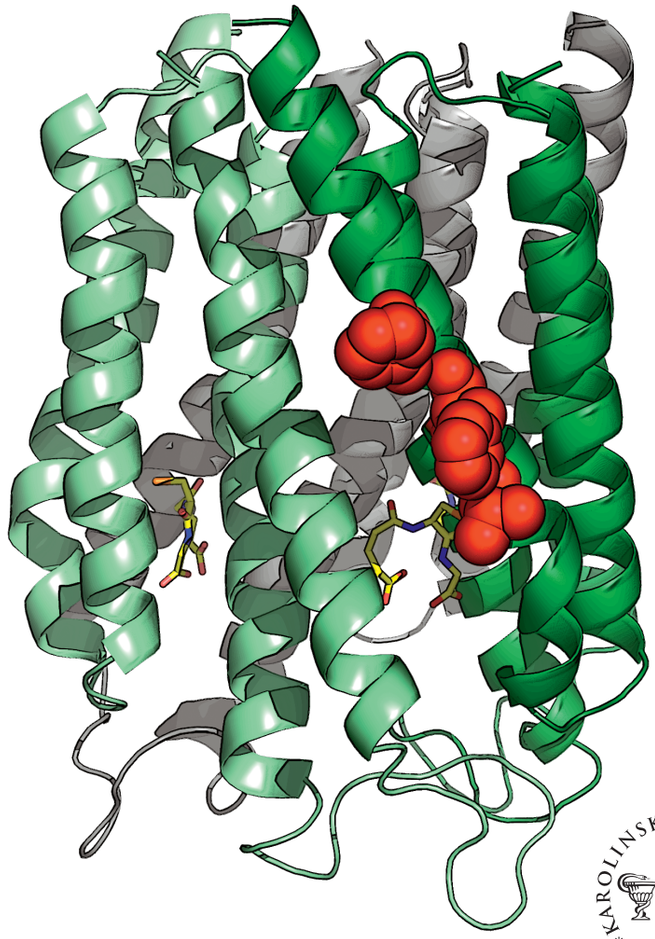

Karolinska Institutet

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Structure and Function of Microsomal Prostaglandin E Synthase-1

Sven-Christian Pawelzik

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Stockholm 2010

Academic dissertation for the degree of Doctor of Philosophy in Medical Sciences at Karolinska Institutet to be publicly defended on Friday, November 26, 2010 at 9:00 in the seminar room of the Centre for Molecular Medicine (CMM), Karolinska University Hospital, L8:00, Stockholm, Sweden.

The defense will be held in English.

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Cover Microsomal Prostaglandin E Synthase-1 (MPGES1) is target to a new generation of non-steroidal anti-inflammatory drugs. In rodent MPGES1, three residues (depicted in red) prevent binding of some of these inhibitors, which have high potency in the human ortholog. This gives evidence that the active site of the homotrimeric protein is located between subunits, close to the co-factor glutathione (depicted in yellow), and that it is accessible from the lipid bilayer.

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“As no better man advances to take this matter in hand, I hereupon offer my own poor endeavors. I promise nothing complete; because any human thing supposed to be complete must for that very reason infallibly be faulty.”

Herman Melville - Moby-Dick; or, The Whale.
Chapter 32

Abstract

The glutathione-dependent enzyme microsomal prostaglandin E synthase-1 (MPGES1) plays a pivotal role in inflammatory diseases. MPGES1 is up-regulated by pro-inflammatory cytokines in concert with cyclooxygenase (COX) -2, and the concerted action of both enzymes leads to the production of induced prostaglandin E_2 (PGE_2), a potent lipid mediator of inflammation, pain, and fever. Non-steroidal anti-inflammatory drugs (NSAIDs) as well as COX-2 specific inhibitors (COXIBs) are widely used analgesics that interfere with PGE_2 production by inhibiting COX. However, use of these drugs is often connected with severe side effects such as gastrointestinal bleeding and cardiovascular events, respectively. This is because these drugs impair the levels of lipid mediators whose formation depends on COX but not on MPGES1. Therefore, specific inhibition of MPGES1 is regarded as a promising strategy in the treatment of inflammatory diseases. MPGES1 inhibitors are currently developed, and it is expected that these novel pharmaceuticals display less severe adverse drug effects while potently eliminating the pro-inflammatory effects of induced PGE_2.

We have conducted studies on the structure and function of MPGES1 in order to understand how this enzyme and its inhibitors work on a molecular level, and the effects of MPGES1 inhibition have been investigated in several disease states. In paper I, the structure of the integral membrane protein MPGES1 was elucidated by electron crystallography. Heterologously expressed human MPGES1 was purified to apparent homogeneity and subjected to two-dimensional crystallisation in the presence of phospholipids. Elastic electron scattering induced by the protein crystals at various angles was used to calculate the three-dimensional structure at 3.5 Å, which was validated by site-directed mutagenesis of structurally and functionally important residues. MPGES1 shows a homotrimeric organisation. Reduced glutathione (GSH), an essential co-factor of MPGES1, binds between two adjacent subunits, but it is not directly accessible from the membrane. Therefore, it is probable that dynamic opening of the protein during the catalytic mechanism allows the substrate PGH_2 to access the active site.

Some of the MPGES1 inhibitors potently block the activity of the human enzyme but do not show any effect on the rat orthologue. In paper II, we exploited this characteristic to investigate the inhibitor binding site of MPGES1. We could change the ability of rat and human MPGES1, respectively, to bind the inhibitor by creating chimeric enzymes. Mutation of single amino acids revealed that three residues, which are aligned at the entrance to the cleft between two adjacent subunits, have a gatekeeper function. The corresponding residues in rat MPGES1 restrict the access for competitive inhibitors to the active site. These results give direct evidence for the location of the active site and

provide a model of how the substrate or competitive inhibitors of the enzyme enter the active site via the phospholipid bilayer of the membrane.

Animal models were used to investigate the effects of MPGES1 inhibition. For the studies in paper III, prostate and lung cancer cells with high constitutive or inducible expression of MPGES1, respectively, were used. Stable knock-down of MPGES1 was established in these cells, which resulted in markedly decreased enzyme activity and slower growth of xenograft tumours in nude mice. Increased apoptosis in response to genotoxic stress was observed, which could be attenuated by exogenous PGE_2. This suggests a role of MPGES1 in tumour progression and beneficial use of specific MPGES1 inhibitors in cancer treatment. In paper IV, we investigated the consequences of MPGES1 deletion after myocardial infarction (MI), which was induced in MPGES1 knock-out mice and wt controls. No difference in infarction size was observed; however, MPGES1 knock-out mice showed worse left ventricular function and altered cardiac architecture 28 days after the event. In both groups prostanoid levels in the tissue were increased to a similar extend after MI, except for PGE_2, which was found to be significantly lower in the knock-out mice. These results imply that MPGES1 derived PGE_2 is important for cardiac tissue remodelling, and deletion of this enzyme results in worse cardiac function after MI. Therefore, use of MPGES1 inhibitors should be carefully considered for patients at cardiovascular risk.

Finally, we evaluated whether the urinary metabolite of PGE_2, tetranor-PGEM, can be used as a biomarker for inflammation. In paper V, we employed LC-MS/MS methodology to quantify tetranor-PGEM without prior derivatisation in the urine from healthy and sick individuals. Levels of tetranor-PGEM remained stable in healthy individuals before and after vaccination, a stimulus of local inflammation associated with mild general symptoms. In patients with fever and active disease, however, tetranor-PGEM levels were elevated compared to healthy controls. The method is sensitive enough to detect baseline levels and will provide a helpful tool for the investigation of inflammatory diseases and the effects of MPGES1 inhibitors.

In conclusion, this thesis provides a deeper understanding of the enzyme MPGES1. The protein structure is presented, the location of the active site was identified, and a mechanism is suggested of how the substrate PGH_2 or competitive MPGES1 inhibitors access the active site from the membrane. These inhibitors may be used as anti-inflammatory drugs, but also as anticancer treatment of certain types of prostate cancer. After MI, however, use of MPGES1 inhibitors might be harmful due to pivotal functions of MPGES1 derived PGE_2 during heart tissue remodelling. In order to analyse depression of systemic levels of PGE_2 caused by MPGES1 inhibitors an analytical method is presented for direct quantification of the urinary PGE_2 metabolite tetranor-PGEM.

List of Publications

This thesis is based on the following previously published papers, which are referred to in the text by their corresponding roman numerals (I to V).

I **Structural basis for induced formation of the inflammatory mediator prostaglandin E_2.**
Jegerschöld C, Pawelzik SC, Purhonen P, Bhakat P, Gheorghe KR, Gyobu N, Mitsuoka K, Morgenstern R, Jakobsson PJ, Hebert H.
Proceedings of the National Academy of Sciences of the United States of America. 2008, 105(32):11110-11115.

II **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.
The Journal of Biological Chemistry. 2010, 285(38): 29254-29261.

III **Microsomal prostaglandin E synthase-1 determines tumor growth *in vivo* of prostate and lung cancer cells.**
Hanaka H, Pawelzik SC, Johnsen JI*, Rakonjac M, Terawaki K, Rasmuson A, Sveinbjörnsson B, Schumacher MC, Hamberg M, Samuelsson B, Jakobsson PJ, Kogner P, Rådmark O.*
Proceedings of the National Academy of Sciences of the United States of America. 2009, 106(44):18757-18762.

IV **Microsomal prostaglandin E_2 synthase-1 deletion leads to adverse left ventricular remodeling after myocardial infarction.**
Degousee N, Fazel S, Angoulvant D, Stefanski E, Pawelzik SC, Korotkova M, Arab S, Liu P, Lindsay TF, Zhuo S, Butany J, Li RK, Audoly L, Schmidt R, Angioni C, Geisslinger G, Jakobsson PJ, Rubin BB.
Circulation. 2008, 117(13):1701-1710.

V **Evaluation of urinary tetranor-PGEM in systemic inflammation.**
Pawelzik SC, Idborg H*, Björk L, Herlenius E, Jakobsson PJ.*
Manuscript.

* These authors contributed equally to the respective study.

Additional Publications

The author of this thesis has also contributed to the following papers and manuscripts, which evolved during the time of his PhD education, but do not form part of the thesis.

- **Lymphoblastic T-cell lymphoma in mice is unaffected by celecoxib as single agent or in combination with cyclophosphamide.**
  Johansson AS, Pawelzik SC, Larefalk Å, Jakobsson PJ, Holmberg D, Lindskog M.
  Leukemia & Lymphoma. 2009, 50(7):1198-203.

- **Hyperforin induces Ca^{2+}-independent arachidonic acid release in human platelets by facilitating cytosolic phospholipase A_2 activation through select phospholipid interactions.**
  Hoffmann M, Lopez JJ, Pergola C, Feisst C, Pawelzik SC, Jakobsson PJ, Sorg BL, Glaubitz C, Steinhilber D, Werz O.
  Biochimica et Biophysica Acta. 2010, 1801(4):462-472.

- **Characterization of a new MPGES1 inhibitor in rat models of inflammation.**
  Leclerc P, Pawelzik SC, Idborg H, Spahiu L, Larsson C, Stenberg P, Korotkova M, Jakobsson PJ.
  Manuscript, submitted to The Journal of Pharmacology and Experimental Therapeutics.

- **Celecoxib and its COX-inactive derivate OSU-03012 sensitize A549 lung cancer cells to vinorelbine-mediated apoptosis independently of PGE_2.**
  Larsson C, Pawelzik SC, Koch A, Wickström M, Stenberg P, Johnsen JI, Sörenson S, Larsson R, Jakobsson PJ, Lindskog M.
  Manuscript, submitted to Cancer Chemotherapy and Pharmacology.

- **Location of inhibitor binding sites within the inducible PGE synthase, MPGES1.**
  Prage EB, Pawelzik SC, Jakobsson, P-J, Morgenstern R, Armstrong RN.
  Manuscript.

- **Effects of selective inhibition of MPGES1 on production of prostanoids and pro-inflammatory mediators by rheumatoid arthritis synovial fibroblasts.**
  Gong L, Sadique S, Pawelzik SC, Stenberg P, Jakobsson PJ, Korotkova M.
  Manuscript.

Popular Science Summary

Most of you who are reading this text right now have probably taken a pill of aspirin at some point of your lives in order to treat pain. Aspirin displays painkilling properties because it interferes with the formation of a signalling substance that sensitises the body for pain. Moreover, this signalling substance plays an important role in the generation of inflammatory diseases and fever. This substance is called Prostaglandin E_2 (PGE_2) and it is produced by the body's cells upon hazardous stimuli. Two enzymes, i.e. biological catalysts, are involved in the production of PGE_2. The first of them, which is known as cyclooxygenase, converts the initial substrate arachidonic acid, into an intermediate molecule, which is called PGH_2. At this step, the second enzyme comes into play and converts PGH_2 into PGE_2. This second enzyme is known under the name microsomal prostaglandin E synthase-1 (MPGES1). Aspirin blocks the first enzyme, cyclooxygenase, at the very beginning of the reaction pathway, so that neither PGH_2 nor the important signalling substance PGE_2 is formed. This explains aspirin's effect as a pain killer and to reduce fever and inflammation.

However, aspirin has further uses. It has, for instance, an antiplatelet effect and prevents the formation of blood clots. In this function it is used to prevent heart attacks. Blood coagulation is mediated by another signalling substance, which also originates from PGH_2 but is formed by another enzyme than MPGES1. If cyclooxygenase is blocked by aspirin, the result is that more than just one signalling molecule will be impaired. This can definitely have very welcome effects, but it can also lead to severe side effects. Especially if patients take aspirin over a long period of time and in high doses, the adverse side effects will prevail. It is therefore desirable to have a better drug that precisely eliminates the pain evoking PGE_2, without affecting the other signalling substances.

Researchers in several pharmaceutical companies are for that reason trying to find an agent that very specifically blocks only the enzyme MPGES1. Such drugs are called MPGES1 inhibitors. I have examined the enzyme MPGES1 in more detail during my postgraduate studies in order to understand the underlying principles of how the newly developed MPGES1 inhibitors work and what consequences it will have to eliminate the functions of MPGES1.

Initially, we aimed to catch a glimpse of how MPGES1 looks like. Together with my collaborators we managed to solve the so called protein structure of MPGES1. By doing so we got a pretty good picture of the enzyme. Based on these results I continued to investigate why there is surprisingly enough a difference in action for some of the MPGES1 inhibitors, depending on whether they act on the human enzyme or on the rat

enzyme during an animal experiment. By changing some of the protein building blocks between the two species I 'humanised' the rat enzyme and 'ratified' the human enzyme. With this approach I was able to determine on a molecular level where the inhibitors bind. In order to study the consequences that inhibition of MPGES1 might have, I have furthermore developed methods that have enabled us to measure tiny amounts of all the different signalling substances originating from PGH_2. Using these methods on biologically and medically relevant samples we discovered that tumours that lost the ability to form PGE_2 grow more slowly than control tumours. Use of MPGES1 inhibitors is here probably beneficial and helpful. One should be careful to use MPGES1 inhibitors, on the other hand, in patients that suffered from a heart infarction, because we also found that PGE_2 plays a role in tissue remodelling of the affected heart.

In conclusion, my thesis work will help to better understand how the newly developed MPGES1 inhibitors work, and it provides a basis to estimate the risks connected with use of these inhibitors.

Populärvetenskaplig Sammanfattning

Förmodligen har de flesta personer som läser den här texten tagit en tablett aspirin någon gång för att behandla smärta. Aspirin förhindrar att en signalmolekyl som gör kroppen känsligare för att förnimma smärtor, bildas och verkar därmed smärtstillande. Signalmolekylen spelar även en viktig roll för uppkomst av inflammatoriska sjukdomar och feber. Denna molekyl heter Prostaglandin E_2 (PGE_2) och bildas i kroppens celler i respons på skadliga retningar. Två enzymer, dvs. biologiska katalysatorer, är involverad i framställningen av PGE_2. Det ena enzymet, som heter cyklooxygenas, omvandlar Arakidonsyra till en molekyl som heter PGH_2. Därefter tar det andra enzymet, mikrosomalt prostaglandin E syntas-1 (MPGES1), över och omvandlar PGH_2 vidare till PGE_2. Aspirin verkar på cyklooxygenaset i början av hela reaktionen, så att varken PGH_2 eller signalmolekylen PGE_2 bildas, vilket förklarar de smärtstillande, febernedsänkande och antiinflammatoriska verkningar av aspirin.

Aspirin har ytterligare egenskaper. Bland annat förebygger aspirin att blodproppar bildas. I blodkoagulationen spelar nämligen en annan signalmolekyl, vilken också härstämmar från PGH_2, en viktig roll. Denna signalmolekyl bildas dock av ett annat enzym i reaktionsvägen än MPGES1. När aspirin hämmar cyklooxygenas blockeras alltså flera signalmolekyler på en och samma gången. Även om man uppnår önskat effekt så kan det också ledar till svåra biverkningar, särkilt när aspirin tas över en mycket lång tid och i höga doser, då de oönskade biverkningarna dominerar för det mesta. Därför vore det bra att ha ett läkemedel som mer specifikt stänger av endast den smärtfrämjade PGE_2, istället för aspirin som påverkar alla andra signalmolekylerna.

På grund av detta försöker forskare från flera läkemedelsbolag sedan några år tillbaka att hitta ett läkemedel som mer specifikt hämmar bara enzymet MPGES1, så kallade MPGES1-inhibitorer. För att förstå vilka mekanismer som ligger bakom inhibering av MPGES1 och vilka följderna blir när man stänger av MPGES1 har jag studerat just detta enzym.

Till en början ville vi få en bild av hur MPGES1 ser ut. Tillsammans med mina medarbetare lyckades vi lösa den så kallade proteinstrukturen, varefter jag fortsatte att utforska varför några av de MPGES1-inhibitorerna som framgångsrikt hämmar det mänskliga enzymet, inte verkar alls på enzymet i råtta vid djurförsök. Jag har ’humaniserat’ enzymet från råtta genom att byta ut några aminosyror från det mänskliga, och sedan har jag, tvärtom, gjort det mänskliga enzymet mer likt enzymet från råttan. På så sätt kunde jag karakterisera var i enzymet inhibitorerna förmodligen binder. För att utforska de fysiologiska följderna av MPGES1 inhibition har jag utöver detta utvecklat metoder som man kan använda för att kvantifiera spårmängder av alla de olika

signalmolekylerna som härstammar från PGH_2. Med hjälp av dessa metoder kunde vi undersöka biologiska och mediciniska relevanta prover och vi upptäckte att tumörer som inte kan bilda PGE_2 växer långsammare än kontrolltumörer. Vid tumörer är det alltså förmodligen fördelaktigt att ta MPGES1-inhibitorer. Å andra sidan måste man dock vara försiktig med MPGES1-inhibitorer efter en hjärtinfarkt, eftersom PGE_2 spelar en roll i ombyggnaden av det skadade hjärtat.

Med resultaten från mitt arbete kan man på molekylär nivå bättre förstå hur de nya MPGES1-inhibitorerna verkar och denna kunskap är viktig för att förstå och uppskatta riskerna i samband med deras användning.

Populärwissenschaftliche Zusammenfassung

Die meisten Leute, die diesen Text lesen, haben vermutlich irgendwann einmal in ihrem Leben eine Tablette Asprin eingenommen, um Schmerzen zu behandeln. Diese schmerzstillende Wirkung hat Aspirin, weil es die Bildung eines Botenstoffs verhindert, der den Körper für Schmerzen sensibilisiert. Darüber hinaus spielt dieser Botenstoff aber auch noch eine wichtige Rolle bei der Entstehung von Entzündungskrankheiten und Fieber. Prostaglandin E_2 (PGE_2), so der Name dieses Botenstoffs, wird vom Körper auf schädigende Reize hin von zwei Enzymen, also von biologischen Katalysatoren, gebildet. Das erste dieser beiden Enzyme, die Cyclooxygenase, wandelt einen Ausgangsstoff namens Arachidonsäure in ein Molekül um, das PGH_2 heißt. Nun kommt das zweite Enzym ins Spiel, das PGH_2 weiter umwandelt in PGE_2. Dieses zweite Enzym heißt Microsomale Prostaglandin E Synthase-1 (MPGES1). Aspirin blockiert die Cyclooxygenase ganz am Anfang des Reaktionsweges, so dass weder PGH_2 noch der Signalstoff PGE_2 gebildet werden. Somit kann man die schmerzstillende, fiebersenkende und entzündungshemmende Wirkung von Aspirin erklären.

Aspirin hat aber auch noch weitere Eigenschaften. Unter anderem beugt es der Bildung von Blutgerinnseln vor. Dies wird nämlich von einem weiteren Botenstoff signalisiert, der ebenfalls von PGH_2 abstammt, dann aber von einem anderen Enzym als MPGES1 gebildet wird. Wenn Aspirin die Cyclooxygenase hemmt, dann werden also gleichzeitig mehrere Botenstoffe beeinträchtigt. Dies kann durchaus erwünschte Wirkungen haben, allerdings auch zu schweren Nebenwirkungen führen. Insbesondere, wenn Aspirin über einen langen Zeitraum und in hoher Dosierung eingenommen wird, dann überwiegen meist die unerwünschten Nebenwirkungen. Daher ist es wünschenswert, ein Medikament zu haben, das das schmerzerzeugende PGE_2 sehr viel gezielter ausschaltet, ohne dass andere Botenstoffe beeinträchtigt werden.

Aus diesem Grund versuchen mehrere Forscher in pharamazeutischen Firmen seit einiger Zeit, spezifische Medikamente zu finden, die nur das Enzym MPGES1 hemmen. Solche Medikamente werden MPGES1-Inhibitoren genannt. Um zu verstehen, nach welchen Prinzipien diese Inhibitoren wirken und welche Auswirkungen es haben kann, wenn man MPGES1 ausschaltet, habe ich in meiner Doktorarbeit dieses Enzym genauer untersucht.

Zunächst einmal wollten wir eine Vorstellung davon bekommen, wie MPGES1 aussieht. Im Team mit meinen Mitarbeitern ist es uns gelungen, die so genannte Proteinstruktur zu entschlüsseln und uns so ein Bild von dem Enzym zu machen. Auf diese Ergebnisse aufbauend habe ich dann untersucht, warum einige MPGES1-Inhibitoren kurioserweise das menschliche Enzym hemmen, aber nicht in Ratten wirken,

wenn man sie im Tierexperiment testen will. Durch den Austausch einzelner Bausteine habe ich das Rattenenzym ‚humanisiert' und das menschliche Enzym dem der Ratte ähnlicher gemacht. So konnte ich die Bindestelle der Inhibitoren charakterisieren. Um die Auswirkungen zu studieren, die eine Hemmung von MPGES1 haben kann, habe ich im weiteren Methoden entwickelt, um kleinste Mengen der verschiedenen von PGH_2 abstammenden Botenstoffe in biologischen Proben zu messen. Mit Hilfe dieser Methoden haben wir biologisch und medizinisch relevante Proben untersucht und herausgefunden, dass Tumore langsamer wachen, wenn sie kein PGE_2 bilden können. Nach einem Herzinfarkt ist allerdings Vorsicht beim Einsatz von MPGES1-Inhibitoren geboten, weil PGE_2 eine Rolle bei der Umgestaltung des geschädigten Herzen spielt.

Meine Arbeit trägt daher dazu bei, besser zu verstehen, wie die neu entwickelten MPGES1-Inhibitoren wirken und bietet eine Grundlage zur Risikoeinschätzung beim Einsatz solcher Inhibitoren.

List of Non-Standard Abbreviations

12-HHT	12(S)-hydroxy-5(Z),8(E),10(E)-heptadecatrienoic acid
13-PGR	15-ketoprostaglandin Δ^{13}-reductase
15-PGDH	15-hydroxy-prostaglandin dehydrogenase
5-HETE	5(S)-hydroxyl-6(E),8(Z),11(Z),14(Z)-eicosatetraenoic acid
5-HPETE	5(S)-hydroperoxy-6(E),8(Z),11(Z),14(Z)-eicosatetraenoic acid
α-linoleic acid	9(Z),12(Z),15(Z)-octadecatrieonic acid
AA	5(Z),8(Z),11(Z),14(Z)-eicosatetraenoic acid, arachidonic acid
APCI	atmospheric pressure chemical ionisation
APPI	atmospheric pressure photoionisation
ATL	15-epi-LXA_4, aspirin-triggered lipoxin
cAMP	cyclic adenosine monophosphate
CID	collision-induced dissociation
COX	cyclooxygenase, prostaglandin H_2 synthase
cPGES	cytosolic prostaglandin E synthase
CRP	C-reactive protein
CYP P450	cytochrome P450
cysLT	cysteinyl leukotriene
DAG	1, 2-diacylglycerol
DGLA	8(Z),11(Z)-14(Z)-eicosatrienoic acid, dihomo-γ-linoleic acid
DMSO	dimethylsulfoxide
EIA	enzyme-immunoassay
EPA	5(Z),8(Z),11(Z)14(Z)17(Z)-eicosapentaenoic acid
ESI	electrospray ionisation
FLAP	5-lipoxygenase activating protein
FTICR	fourier transform ion cyclotron resonance
GC-MS	gas chromatography coupled to mass spectrometry
GSH	reduced glutathione (γ-glutamylcysteinglycine)
GPCR	G protein-coupled receptor
H-PGDS	haematopoietic PGD Synthase
Hsp	heat shock protein
IP_3	inositol 1, 4, 5-trisphosphate
LDLR	low density lipoprotein receptor
linoleic acid	9(Z),12(Z)-octadecadienoic acid
LO	lipoxygenase
LTC4S	leukotriene C_4 synthase
m/z	mass to charge ratio
MAPEG	membrane associated proteins in eicosanoid and glutathione metabolism

MDA	malondialdehyde
MGST	microsomal glutathione transferase
MI	myocardial infarction
MPGES	microsomal prostaglandin E synthase
MRM	multi reaction monitoring
NMR	nuclear magnetic resonance
NEM	N-ethyl-maleimide
L-PGDS	lipocalin-type PGD synthase
LC-MS	liquid chromatography coupled to mass spectrometry
LPS	lipopolysaccharide
LT	leukotriene
LX	trihydroxyeicosatetraenoic acid, lipoxin
PDB	protein data bank
PG	prostaglandin
PGHS	prostaglandin H_2 synthase, cyclooxygenase
Q	quadrupole
QQQ	triple-quadrupole
RIA	radio-immunoassay
RP-HPLC	reversed-phase high-performance liquid chromatography
RSV	respiratory syncytial virus
TBA	2-thiobarbituric acid
tetranor-PGEM	13,14-dihydro-15-keto-2,3,4,5-tetranor-prostan-1,20-dioic acid
TM	transmembrane helix
TOF	time-of-flight
TX	thromboxane
TXAS	thromboxane A_2 synthase
wt	wild type

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Introduction

A Brief History of Pain Relief

Pain is a fundamental experience of life, and ever since mankind has had to face pain, it has been looking for a remedy. The earliest known references trace back to the ancient Egyptians. In one of the oldest and most important medical papyri, the Ebers Papyrus, which dates back to the reign of Amenhotep I around 1534 BC, the medicinal use of myrtle and willow tree bark, original sources of aspirin-like compounds, was recommended for the treatment of stiff and painful joints. This Egyptian knowledge influenced ancient Greek physicians, among them the most well known Hippocrates of Cos (460-377 BC), who spent several years in Egypt studying medicine. He noted that chewing of the bitter leaves of the willow tree reduces pain (1).

Modern pharmaceutical medicine, which tries to investigate the molecular basis of disease and the mechanisms by which traditional cures obtained from natural products work, began to evolve in the 19th century. In 1828 Johan Andreas Buchner first isolated salicin from willow bark, a compound named after its source, *Salix alba*, the white willow. Raffaelle Piria treated salicin in 1838 to yield salicylic acid, which was found to possess profound medicinal properties and was soon widely used for all kinds of disorders, despite the severe side effects of gastric irritation, bleeding and diarrhoea (1). A French chemist, Charles Frederic Gerhardt, was in 1853 the first one to produce "salicylic-acetic anhydride" from acetyl chloride and the sodium salt of salicylic acid (2). However, he did not pay attention to the medical aspects of his discovery and hence did not further pursue this product and its improved gastro-intestinal properties. Therefore, his discovery sank into oblivion for almost 50 years, until Felix Hoffmann, a German chemist who was working for the drug and dye company Bayer, re-discovered and perfected Gerhardt's formulation to acetylate salicylic acid in 1897. Hoffmann tried acetylsalicylic acid for the first time on his father, who suffered from arthritic pain, and after this turned out to be successful, he convinced Heinrich Dreser, the head of Bayer's pharmaceutical division, to investigate this substance in animal experiments and subsequently also on patients (3). On March 6, 1899, the substance was patented under the name 'aspirin', derived from 'a' for acetyl, 'spir' for *Spirsäure*, an old German name for saclicylic acid based on the latin name for meadowsweet, *Spiraea ulmaria*, and 'in' as a then-typical suffix for medicine.

In an attempt to produce codeine, a constituent of the opium poppy and a popular cough remedy, Felix Hoffmann also acetylated morphine and hence developed another analgesic drug, diacetylmorphine, which he called 'heroin'. The name is derived from the perceived 'heroic' effect it had upon volunteers testing it. From 1898 on, heroin was massively marketed as an apparently non-addictive morphine substitute and a cough suppressant. It was furthermore indicated for pain relief and as such marketed side by side with aspirin. When it became clear in 1904 that orally administered heroin is metabolised via deacetylation to 6-acetylmorphine and morphine, making it a prodrug for the systemic delivery of morphine, and furthermore that heroin, administered by smoking or by direct injection into the circulation, passes the blood-brain-barrier much more rapidly than morphine itself, which makes it even more addictive, its use was more and more stigmatised (4). However, it was not until 1910 distribution was ceased for this once-freely available and very popular drug. In fact, Bayer continued the production of heroin until 1931. This development paved the way for the success of aspirin as the best available analgesic drug at the beginning of the 20th century, and promotion enforced by Bayer established the brand name worldwide.

The mechanism, by which aspirin works, however, remained unknown. Nonetheless, two lines of research helped to solve this puzzle. The biochemistry involved was largely investigated by Swedish researchers. At the beginning of the 20th century physiologists recognised that extracts from human prostate glands had effects on the blood pressure and the urinary bladder of dogs (5). Furthermore, it was discovered that human semen induces the uterus to contract (6). Based on these observations Ulf Svante von Euler (7-9) and Maurice Walter Goldblatt (10) independently discovered other substances in human seminal plasma with profound physiological activities. Because it appeared that these substances originated from the prostate gland, they were coined 'prostaglandin' in 1935 (8). Von Euler made contact with Sune Karl Bergström regarding these prostaglandins. Bergström, a biochemist at Karolinska Institutet, accomplished further research, and together with Jan Sjövall he isolated two compounds from sheep seminal vesicles, one of which was soluble in ether (11) and the other one in phosphate buffer (12). Based on the Swedish words for the solvents, *eter* and *fosfat buffer*, the two compounds were named prostaglandin E and prostaglandin F, respectively. These findings, and the subsequent structural elucidation of the prostaglandins E and F (13) by Sjövall, Bergström and his graduate student Bengt Ingmar Samuelsson, led to the discovery of a whole family of biologically active substances, the eicosanoids. Among the most notable studies during the following decades was the discovery that all of these biologically active substances originate from polyunsaturated fatty acids with 20 carbon atoms, predominantly from arachidonic acid (AA, 5(Z),8(Z),11(Z),14(Z)-eicosatetraenoic acid, 20:4 ω6). This was independently shown by Bergström (14) and David A. van Dorp (15-16) and led to the name "eicosanoids", based on the Greek word *eikosi* for twenty. Samuelsson continued to work on the biochemical pathway of prostaglandin formation and postulated a cyclic endoperoxide to be involved in the reaction, leading to prostaglandin formation (17). The enzyme forming this endoperoxide was termed prostaglandin H synthase, or cyclooxygenase (COX). Together with Mats Hamberg he isolated two short-lived

endoperoxides, prostaglandin (PG) G_2 (18) and PGH_2 (19), the conversion products of AA by COX, which are crucial intermediates in the pathway leading to PGE_2 and $PGF_{2\alpha}$. Shortly thereafter Hamberg and Samuelsson also discovered the eicosanoid thromboxane A_2 (TXA_2), which induces blood clotting (18, 20).

The biochemical work on prostaglandins was complemented with results from physiological studies that the British pharmacologist John Robert Vane accomplished at the same time. Vane was interested in the release and fate of vasoactive hormones in the circulation and focused on prostaglandins in the mid-1960s. He was working with the development of bioassays, a method by which the effect of different compounds and drugs on an organ can be studied. This method enabled him to prove that prostaglandins are produced by many tissues and organs in the body, not only by the prostate gland as initially assumed (21). In one of his experiments he tested the effect of aspirin and found it to inhibit the production of a PGE_2 (22). With this observation he established the missing link between aspirin, prostaglandins, and pain relief. This concept was later proven when it was shown that aspirin inhibits COX activity by acetylation of a specific serine residue within the active site of the enzyme (23-24). A few years later, after the discovery of TXA_2 by Hamberg and Samuelsson, Vane found a new member of the eicosanoid family with properties opposite to that of TXA_2, inhibiting blood clot formation (25), which he initially named prostaglandin X and later re-named prostacyclin (PGI_2).

"For their discoveries concerning prostaglandins and related biologically active substances" (26) Sune K. Bergström, Bengt I. Samuelsson and John R. Vane were jointly awarded the Nobel Prize in Physiology or Medicine in 1982.

Today it is known that COX converts AA to yield PGH_2, and by doing this it provides the substrate for several terminal and specific prostaglandin synthases, leading to the formation of TXA_2, PGI_2, PGE_2, PGD_2, and $PGF_{2\alpha}$. Inhibition of COX by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) affects all of these prostanoids, including PGE_2 and PGI_2, which are both involved in pain perception. This is how NSAIDs work as painkillers. However, this clear-cutting also leads to the side effects that are connected with this class of drugs, namely gastrointestinal events. It is expected that a more fine-tuned inhibition that targets only the terminal synthase leading to PGE_2 will have a more beneficial effect profile. Prostaglandin E synthase activity was observed early on in microsomes from bovine and sheep vesicular glands (27-29). However, it was not until 1999 that cloning and expression of microsomal prostaglandin E synthase-1 (MPGES1) was described by Per-Johan Jakobsson and his co-workers (30). MPGES1 is up-regulated by pro-inflammatory cytokines and has been shown to be associated with induced PGE_2 production during inflammation, pain and fever. Therefore, it is regarded as a promising drug target, and specific inhibitors are under development.

Aim of This Study

When this dissertation work started, MPGES1 has been characterised in terms of its substrate requirements, its catalytic activities, and its affiliation to the protein superfamily of membrane associated proteins involved in eicosanoid and glutathione metabolism (MAPEG). Furthermore, its role in inflammatory diseases, especially in the inflammatory autoimmune disease rheumatoid arthritis, was established. However, only basic facts were known about structural features of the enzymes belonging to the MAPEG superfamily in general and of MPGES1 in particular. Based on bioinformatic techniques, such as hydropathy plots, four transmembrane helices (TM) were predicted for MPGES1 (31-32). However, because these techniques rely on theoretical models, only three helices were assigned for some of the MAPEG members (33). Early structural investigation using electron crystallography and hydrodynamic studies pointed to a trimeric arrangement of MPGES1 (34), but biochemical data predicted dimers for other MAPEG members (35-36).

The first aim of the work included in this thesis was therefore to determine the protein structure of MPGES1 in order to gain insight into the mechanism, the substrate binding and the interaction with specific inhibitors.

These inhibitors were in the early stages of development at the time this work began. Using them as a powerful tool in combination with the protein structure, the second aim of this thesis, the investigation of the inhibitor binding site and the active site of MPGES1, could be achieved.

Mice deficient in the gene that codes for MPGES1 were developed in 2003 in order to study the consequences of targeted interference with PGE_2 formation (37). Also, several methods for the quantitative analysis of eicosanoids employing liquid chromatography separation and detection by mass spectrometry (LC-MS/MS) have been developed.

The third aim of this study was to establish these analytical techniques in our laboratory and refine them to be suited for the needs of specific projects, considering analytes of interest, work-up of different biological material, and sensitivity of the method.

Altogether, the overall aim of this dissertation work was to study the molecular basis of how the enzyme MPGES1 and newly developed MPGES1 inhibitors work, and to investigate the consequences that inhibition of this enzyme might have.

Background

Eicosanoids

The Eicosanoids form a widespread family of signalling molecules that are derived from polyunsaturated fatty acids. The major groups of the eicosanoid family are the prostanoids, including prostaglandins, prostacyclins, and thromboxanes, and the leukotrienes (Figure 1). All of these compounds consist of a chain of 20 carbon atoms, hence the name of the group, which is based on the Greek word *eikosi*, meaning twenty. Eicosanoids are hormone-like molecules that have profound physiological effects at very low concentrations. Unlike hormones, however, they are inactivated within seconds or minutes because of their chemically and biologically unstable nature. Therefore, they are not transported by the circulation and do not conduct systemic signals. Rather, they act as local, autocrine or paracrine mediators in the same environment in which they are synthesised. Furthermore, and in contrast to the more uniform actions of global hormones, the effects of eicosanoids vary from one type of cell to another.

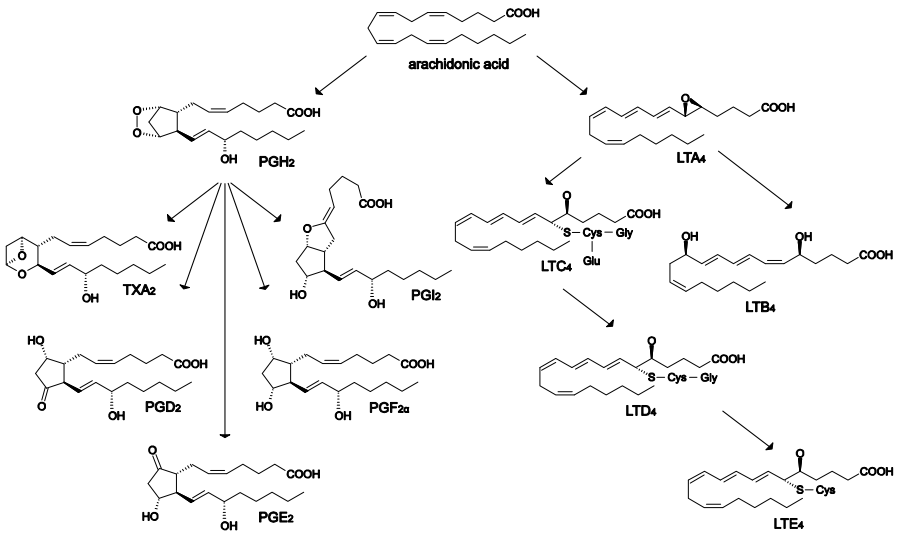


Figure 1. Biosynthesis of prostanoids and leukotrienes. *Arachidonic acid can be metabolised to prostaglandins (PGE_2, PGD_2, and $PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxane (TXA_2) via the COX pathway and to leukotrienes (LTA_4 and LTB_4) and cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) via the 5-LO pathway.*

A variety of polyunsaturated fatty acids can be formed by mammals *de novo*. Newly synthesised fatty acids can be modified by a combination of elongation and desaturation reactions. However, mammalian cells lack the enzymes that are necessary to introduce double bonds beyond C-9 in a fatty acid chain. Therefore, two essential fatty acid need to be supplied with the diet: α-linoleic acid (9(Z),12(Z),15(Z)-octadecatrieonic acid, 18:3 ω3) and linoleic acid (9(Z),12(Z)-octadecadienoic acid, 18:2 ω6). The major precursor of eicosanoids in humans is AA, a C-20 polyunsaturated fatty acid with four non-conjugated carbon-carbon double bonds at C-5, C-8, C-11, and C-14. Because two of these double bonds are beyond C-9, the only way AA can be formed by mammals is via linoleic acid. Further metabolised, AA will give rise to prostanoids with two carbon-carbon double bonds, the series-2 prostanoids. The number of double bonds is denoted by a subscript in the name of every species, e.g. PGE_2 (38). If linoleic acid is modified to become a C-20 polyunsaturated fatty acid with only three double bonds, dihomo-γ-linoleic acid (DGLA, 8(Z),11(Z),14(Z)-eicosatrienoic acid, 20:3 ω6), prostanoids with only one carbon-carbon double bond will arise. α-linoleic acid can be metabolised to yield eicosapentaenoic acid (EPA, 5(Z),8(Z),11(Z)14(Z)17(Z)-eicosapentaenoic acid, 20:5, ω3), which can be further metabolised to yield prostanoids with three carbon-carbon double bonds. Figure 2 gives an overview of the types of prostanoids that are formed from different precursors.

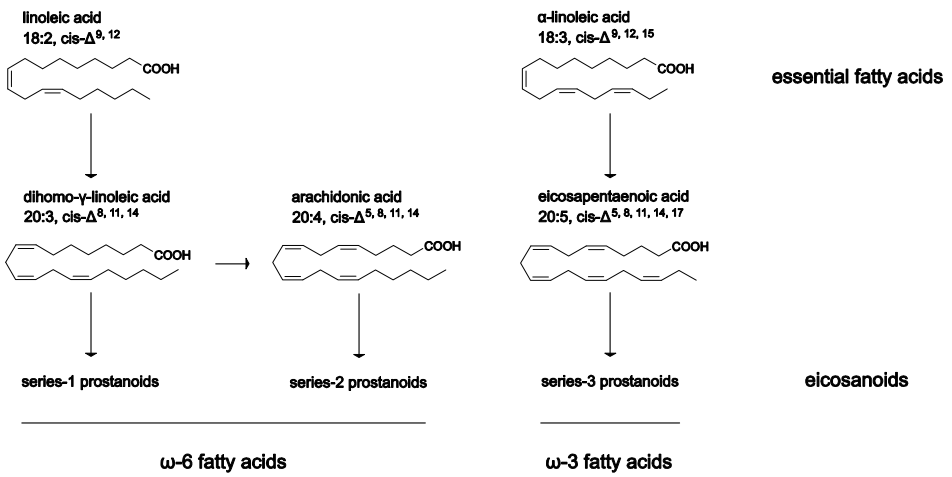


Figure 2. Precursors of eicosanoids. *Linoleic acid and α-linoleic acid are essential fatty acids that need to be supplied with the diet. They can be metabolised to yield dihomo-γ-linoleic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA), which will give rise to series-1, series-2, and series-3 prostanoids, respectively.*

Release of Arachidonic Acid

Under normal conditions the concentration of free AA within a cell is low. Most of it is stored as part of phospholipids in the membranes of the cell. AA is usually esterified at the *sn*-2 position of the glycerol molecule present in phosphatidylinositol and other phospholipids (39). From there it can be released upon cell activation by the action of phospholipase enzymes. Because availability of free AA is essential for the biosynthesis of eicosanoids, its release is regulated by the activation of G protein-coupled receptors GPCRs (40), phosphorylation of phospholipases (41), as well as intracellular Ca^{2+} levels (42). The levels of free AA are furthermore tightly controlled by the opposite action of phospholipases and acyl-CoA transfereases, which liberate and re-esterify AA, respectively (43).

Phospholipase enzymes are grouped according to their substrate specificity: Phospholipase A_2 and Phospholipase D can use phosphatidylcholine as a substrate and directly hydrolyse acyl groups at the *sn*-2 position to yield lysophospholipids and AA, while Phospholipase C specifically hydrolyses the head groups of phosphatidylinositol to yield the second messenger molecules 1, 2-diacylglycerol (DAG, with stearic acid at *sn*-1 position and AA at *sn*-2 position) and inositol 1, 4, 5-trisphosphate (IP_3) (40). DAG can in turn either be directly hydrolysed by Diacylglycerol Lipase to release AA, or it may be phosphorylated to yield phosphatidic acid, which serves as a substrate for Phospholipase A_2 for the release of AA (40).

Several isozymes of Phospholipase A_2 have been identified so far. They can be classified into secretory, extracellular forms ($sPLA_2$) with a low molecular weight (14-17 kDa) and intracellular forms with a high molecular weight (>60 kDa). The cytosolic forms of Phospholipase A_2 can be further subdivided into Ca^{2+}-dependent forms ($cPLA_2$) and Ca^{2+}-independent forms ($iPLA_2$). Apart from these enzymes, they also exist as lipoprotein-associated Phospholipase A_2 enzymes, which are also known as Platelet-Activating Factor Acetyl Hydrolases (44). Phospholipase enzymes are globular, soluble proteins that are translocated to the membrane upon cell activation, where they become active. Corticosteroids, which are known as potent anti-inflammatory agents, down-regulate the expression of phospholipase A_2 enzymes and thus reduce the production rate of free AA and their subsequent lipid mediators of inflammation (45-46).

Although many phospholipases can release AA, $cPLA_2$-α has been shown to be of particular importance for the generation of eicosanoid mediators. $cPLA_2$-α is constitutively expressed in most cells and tissues, but its expression can be induced under certain conditions (47). $cPLA_2$-α preferentially releases AA from the *sn*-2 position of phospholipids (48), but it also possesses *sn*-1 lysophospholipase activity and weak transacylase activity (49). Submicromolar concentrations of Ca^{2+} are required for the translocation of $cPLA_2$-α from the cytosol to the membrane of the nuclear envelope (50). Studies using mice deficient in the gene coding for $cPLA_2$-α have confirmed its role in the biosynthesis of eicosanoids (51). Pregnancy in female $cPLA_2$-α knock-out mice was less frequent compared to wt mice, and the pups had an increased mortality rate. The mice had

lesions in the small intestine and impairments to concentrate the urine when being water-deprived. Mast cells and peritoneal macrophages isolated from these mice showed a decreased capacity to produce eicosanoid mediators; however, it was possible to quantify prostaglandins and leukotrienes from these cells after stimulation for 12 hours. It can be interpreted that $cPLA_2$-α is involved in the acute release of AA metabolites (51). In different disease models, including acute lung injury, anaphylaxis (52), and experimental Parkinson syndrome (53), $cPLA_2$-α knock-out mice were less responsive than the wt controls.

One human subject has been reported to carry an inherited mutation of $cPLA_2$-α (54).This resulted in platelet dysfunction, impaired biosynthesis of TXB_2 and 12-hydroxyeicosatetraenoic acid (HETE), decreased synthesis of leukotrienes, and ulceration of the small intestine. Therefore, $cPLA_2$-α apparently has a similar role in mice and men.

The protein structure of $cPLA_2$ (PDB code 1CJY) shows that this 85 kDa enzyme consists of two domains, an N-terminal C2-domain mainly formed by β-sheets, and an α-helical C-terminal catalytic domain (55). The C2-domain is able to bind two Ca^{2+} ions and is comprised of a patch of basic residues involved in membrane binding. Within the catalytic domain leads a funnel structure to the active site of $cPLA_2$-α. The enzyme works as a serine hydrolase with Ser-228 as the catalytic residue, which is activated by Asp-549. Residue Arg-200 is believed to stabilise the phosphate group of the phospholipid substrate. The architecture of the funnel structure is believed to confer the strong preference of $cPLA_2$-α for AA (56). Figure 3 shows the protein structure of cPLA2.

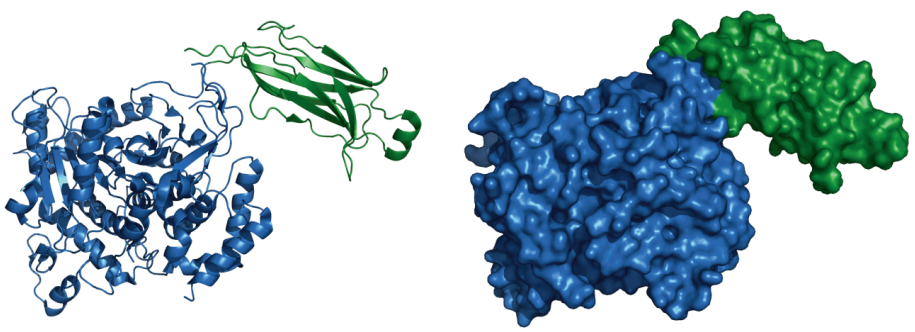


Figure 3. Crystal structure of human cytosolic phospholipase A_2. *The structure of $cPLA_2$ was solved by x-ray crystallography at 2.5 Å (PDB code 1CJY). The protein is depicted in cartoon representation (left) and in surface representation (right). The N-terminal C2-domain, which is mainly consisting of β-sheets, is shown in green, and the α-helical, C-terminal catalytic domain is shown in blue.*

Metabolism of Arachidonic Acid

When AA is released from the membrane, it is rapidly oxygenated by different enzyme systems to yield lipid mediators of the eicosanoid family. The specific type of mediator that is produced is largely dependent on the set of enzymes expressed in the cells of a specific tissue. Several enzymatic pathways are known to catalyse oxygenation of AA.

The first pathway of AA metabolism is based on the action of cyclooxygenase (COX). It involves the cyclic endoperoxide intermediates PGG_2 and PGH_2 and yields the prostanoid mediators, i.e. the three prostaglandins E_2, D_2, and $F_{2\alpha}$, prostacyclin, and thromboxane. This pathway is most important for the studies included in this thesis and will be therefore described in detail below.

The second pathway depends on the catalytic activity of 5-lipoxygenase (LO) and leads to the formation of leukotrienes. 5-LO is a soluble, monomeric enzyme of 78 kDa that resides in the cytosol. The structure of human 5-LO has recently been elucidated (57), but it is not yet published or deposited in the protein data base (PDB). However, it was reported to display a high degree of similarity to the structure of 15-LO-1 from rabbit reticulocytes (PDB code 1LOX), the only full length three-dimensional protein structure available for mammalian lipoxygenases (58). It consists of two domains, an N-terminal membrane binding domain and a highly conserved C-terminal catalytic domain that contains a non-haem iron at the active site of the enzyme (58). Upon cell activation and subsequent increase in the intracellular concentration of Ca^{2+}, 5-LO translocates to the membranes of the nuclear envelope and the perinuclear endoplasmic reticulum. Activated 5-LO is thus situated close to the site of AA release, but it is not able to utilise AA as a substrate unless it interacts with an integral membrane protein, 5-lipoxygenase activating protein (FLAP). By a yet unknown mechanism FLAP makes AA available for 5-LO, which then catalyses the oxygenation of AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and the subsequent conversion to the unstable epoxide leukotriene (LT) A_4. LTA_4 is the precursor for all other leukotrienes. It can be hydrolysed by LTA_4 Hydrolase to give rise to the potent chemotactic agent LTB_4, or conjugated with reduced glutathione (GSH) by LTC_4 Synthase (LTC4S) to yield LTC_4. LTC_4 can be further modified by sequential removal of glutamic acid and glycine from the GSH moiety to yield LTD_4 and LTE_4, respectively. LTC_4, LTD_4, and LTE_4 together are known as cysteinyl leukotrienes (cysLTs) and comprise the pathophysiological activities that were previously attributed to the so-called ‘slow reacting substance of anaphylaxis’ (59).

Leukotrienes owe their names to the fact that they are predominantly formed by leukocytes and that all of these molecules contain three conjugated double bonds (38). They are potent pro-inflammatory mediators and play a pivotal role in asthmatic diseases. There are some medications for the treatment of asthma on the market that interfere with the synthesis or the action of leukotrienes (60-61). Zileuton, known under its trade name Zyflo, is an inhibitor of 5-LO and interferes directly with the biosynthesis of LTA_4. Zileuton is used for the maintenance treatment of asthma. A different strategy is used by the two drugs Montelukast and Zafirlukast, which are marketed under the trade names

Singulair and Accolate, respectively. These drugs are selective antagonists of the cysteinyl leukotriene receptor $CysLT_1$ in the lungs and the bronchial tubes. $CysLT_1$ is the main receptor for LTD_4, but it is also activated by LTC_4 and LTE_4, albeit to a lower extent. Montelukast and Zafirlukast bind to $CysLT_1$ but do not provoke biological response. This reduces the bronchoconstriction otherwise caused by the cysLTs, and results in less inflammation. Montelukast and Zafirlukast are used for the maintenance treatment of asthma and to relieve symptoms of seasonal allergies. Apart from these drugs, which are readily available on the market, a third class of pharmaceuticals, the FLAP inhibitors, exists. These exploit the fact that FLAP is indispensable for leukotriene biosynthesis. By inhibiting FLAP they interfere indirectly with the biosynthesis of LTA_4. Several FLAP inhibitors are currently in clinical development for treatment of respiratory and atherosclerotic diseases (62-63). A novel strategy for the treatment of asthma is the specific inhibition of LTC4S (61).

In addition to these two classical pathways of eicosanoid biosynthesis leading to prostanoids and leukotrienes free AA can also be metabolised by other lipoxygenases like 15-LO-1 and by certain members of the Cytochrome P-450 family, respectively, which are key enzymes in pathways that lead to the so called nonclassic eicosanoids.

15-LO-1 catalyses the oxygenation of a variety of lipids. In contrast to 5-LO, which strongly prefers free AA over other polyunsaturated fatty acids, 15-LO-1 oxygenates several polyunsaturated fatty acids regardless of their chain length, even fatty acids that are esterified in phospholipids and incorporated in biomembranes and lipoproteins (64). The protein structure of human 15-LO-1 is expected to be highly similar to the structure of rabbit 15-LO-1 described above. Furthermore, because the amino acids around the non-haem iron at the active site are conserved (65-66), the catalytic mechanism is generally believed to be the same for all mammalian lipoxygenases. However, there are certain changes in the active site of the various lipoxygenases affecting its size and hydrophobicity and resulting in different substrate specificities and product profiles. 15-LO-1 catalyses the conversion of free AA to the intermediate peroxide 15(S)-HPETE (67), which is subsequently reduced to give rise to 15(S)-HETE, the main product of 15-LO-1. Analogous to the reaction catalysed by 5-LO to yield LTA_4 via 5-HPETE, 15-LO-1 can also catalyse the generation of eoxin A_4 (EXA_4) via 15(S)-HPETE (68). Subsequently, EXA_4 can be used as a substrate for LTC4S and conjugated with GSH to yield EXC_4, which then can be further metabolised to EXD_4 and EXE_4 (69). The 15-LO-1 pathway is summarised in Figure 4.

Some members of the cytochrome P450 (CYP P450) superfamily are also able to metabolise AA and are hence involved in the CYP P450 pathway. In contrast to COX and lipoxygenase enzymes, which all are dioxygenases, CYP P450 enzymes possess monooxygenase activity and catalyse the redox-coupled activation of molecular oxygen and the subsequent delivery of one oxygen atom to the lipid substrate while the other oxygen atom is reduced to water (70). Oxidation of AA by CYP P450 generates different HETEs or epoxyeicosatrienoic acids (EETs).

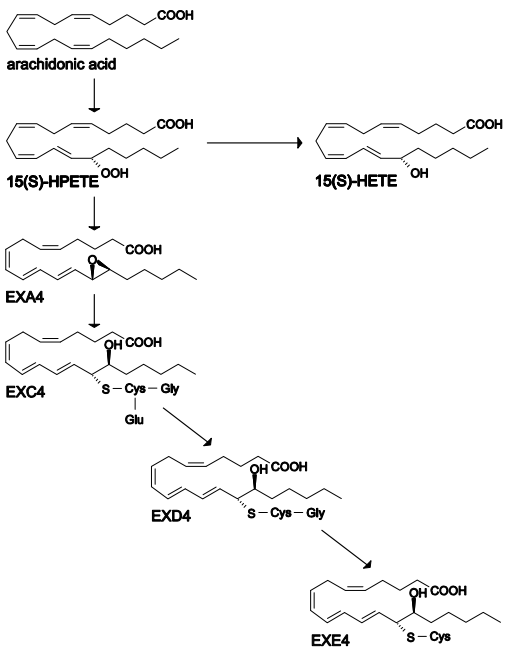


Figure 4. Biosynthesis of 15(S)-HETE and eoxins. *15-LO-1 catalyses the conversion of arachidonic acid to 15(S)-HPETE, which can be reduced to 15(S)-HETE, or further metabolised into eoxins.*

The enzyme systems leading to eicosanoid production are usually expressed within the same cell and form tight biosynthetic complexes that assemble at the membrane, such as $cPLA_2$, 5-LO, and FLAP (61). However, in some instances, the reaction is not so strictly regulated, and intermediates may be transferred between various cells that express different enzymes. This so called transcellular metabolism of eicosanoids leads to the formation of lipoxins.

The lipoxins (LX) comprise different trihydroxyeicosatetraenoic acids with anti-inflammatory properties that may play a role in the resolution of inflammation. Three putative transcellular pathways in the biosynthesis of lipoxin A_4 (LXA_4) have been characterised (71). The first of these pathways depends on the interaction of leukocytes and platelets. AA is liberated in leukocytes and converted to LTA_4 by 5-LO. LTA_4 is then released from the leukocytes and taken up by adherent platelets, where it is transformed by another lipoxygenase with different specificities, 12-LO, resulting in LXA_4 (72-73). The second known pathway that gives rise to lipoxins is initiated in epithelial or endothelial cells expressing 15-LO-1. This enzyme converts AA to 15(S)-HETE, which is subsequently taken up by leukocytes, usually polymorphonuclear neutrophils (PMNs), expressing 5-LO and converting 15(S)-HETE into LXA_4 (74). Finally, COX-2, an enzyme that is classically involved in the prostanoid pathway of AA metabolism and is expressed in epithelial or endothelial cells, is able to produce 15(R)-HETE when it is acetylated by aspirin. This intermediate can be taken up by leukocytes and further metabolised by 5-LO to 15-epi-LXA_4, also known as aspirin-triggered lipoxin, or ATL (74).

The Cyclooxygenase Pathway

All prostanoids contain a five- or six-membered ring structure within their carbon chain, in contrast to the linear carbon chain of the other eicosanoids. PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 include a cyclopentane ring, while the structurally related TXA_2 is characterised by a pyran ring. This cyclic structure is already present in the immediate precursor of all of these molecules, PGH_2, and results from the enzymatic activity of Prostaglandin H Synthase. PGHS is a homodimeric enzyme with a molecular weight of approximately 70-72 kDA, and converts free AA to PGH_2 in a two-step sequential reaction. It has two distinct catalytic centres that catalyse a haem-dependent bis-oxygenase reaction and a peroxydase reaction, respectively. Due to the first of these activities, PGHS is often denoted by its common name cyclooxygenase, or COX.

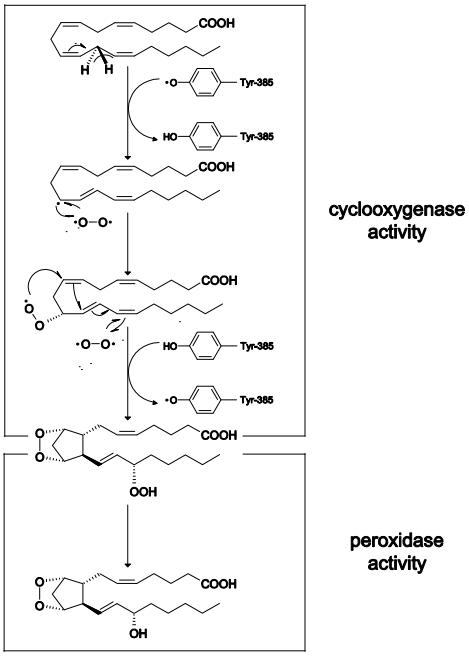


Figure 5. The mechanism of PGH_2 biosynthesis. *COX contains two active sites. At the cyclooxygenase active site of the enzyme arachidonic acid is oxidised in a radical mechanism to yield PGG_2, which is subsequently reduced at the peroxidase active site of COX.*

The cyclooxygenase activity of COX catalyses the oxidation of AA with two molecules of oxygen; leading to the formation of the endoperoxide PGG_2. The hydroperoxide group at carbon 15 of PGG_2 is subsequently reduced to a hydroxyl group by the peroxidase activity of COX (75-78).

The mechanism of PGH_2 biosynthesis (Figure 5) was outlined by Hamberg and Samuelsson in 1967 (79-80) and subsequently verified by mutagenesis studies and the determination of the molecular crystal structure in 1994 (76, 81). The substrate is bound by COX and properly positioned by hydrogen bond interactions between the carboxyl group of AA and the amino acid side chain of Arg-120. Using a tyrosyl radical at position 385 that is generated by the haem cofactor, the enzyme oxidises AA by removing the 13-pro-S-hydrogen atom from AA. This produces a carbon radical at position 11 of AA, which in turn reacts with molecular oxygen to generate a peroxyl radical. This radical adds to carbon 9 to form a cyclic peroxide and a new carbon radical at carbon 8. Rearrangement of electron pairs generates the cyclopentane ring and a radical at carbon 15. A second molecule of oxygen is introduced at the position of this radical, which produces another peroxyl radical and finally abstracts the hydrogen from Tyr-385. Thus,

the tyrosyl radical at the cyclooxygenase active site of the enzyme is recycled and PGG_2 is formed. PGG_2 then moves to the peroxidase active site of COX, where its hydroperoxyl group at carbon 15 is reduced to a hydroxyl group, which yields PGH_2.

Two isoforms of COX have been found in mammals, referred to as COX-1 (82-84) and COX-2 (85-86). Although the two isozymes are closely related to each other and display a sequence identity of about 65% on the amino acid level, they differ markedly in their expression pattern and function. COX-1 is in most cases constitutively expressed in many tissues and considered to maintain housekeeping functions involved in homeostasis, such as regulation of renal blood flow, platelet function, and maintenance of the gastric mucosa. In contrast to this, COX-2 is not detectable in most quiescent cells, but it can be induced by a variety of inflammatory stimuli (87). The promoter of the COX-2 gene contains various putative regulatory elements that control its transcription. COX-2 is expressed in response to lipopolysaccharide (LPS), pro-inflammatory cytokines like interleukin-1, tumour necrosis factor-α, and interferon-γ, hormones, such as follicle-stimulating hormone, luteinising hormone, and estrogen, and the growth factors EGF, PDGF and FGF (88). Over-expression of the COX-2 gene is associated with fever as well as with several disorders, such as inflammatory diseases, various types of cancer, and some neurological disorders.

Both isozymes are located at the membranes of the endoplasmatic reticulum and the nuclear envelope, however, the subcellular distribution of the two enzymes differs. As shown by studies using immunocytological techniques and confocal fluorescence microscopy, COX-1 is predominantly located at the membrane of the endoplasmatic reticulum, whereas the concentration of COX-2 is higher at the membrane of the nuclear envelope (89).

In contrast to other membrane-associated proteins, COX is not embedded in the lipid bilayer of the membrane. Instead, it uses the hydrophobic surfaces of a set of amphipatic α-helices that extend from the bottom of the protein to attach to the membrane. This linkage is sufficiently strong that only the action of detergents can release the protein. Thus, this enzyme is classified as an integral membrane protein, although it is not membrane-spanning.

The protein structures of ovine COX-1 (PDB code 1CQE) (90) and murine COX-2 (PDB code 6COX) (91) were solved by x-ray crystallography in 1994 and 1996, respectively. They show that both enzymes are homodimeric proteins that are primarily made up of α-helices and glycosylated at several points. Each monomer has its own set of catalytic centres and consists of three structural domains: an N-terminal EGF-like domain promoting dimerisation, a membrane binding domain that comprises the above mentioned amphipatic α-helices, and a large, globular catalytic domain at the C-terminus that includes the binding site for the haem group, the glycosylation sites, and a KDEL-like sequence that may target the enzyme to the endoplasmic reticulum.

The crystal structure of COX also revealed information about the catalytic centres of the enzyme (90). The peroxidase active site, indicated by the prosthetic group, a high-spin Fe(III)-protoporphyrin IX haem, is located at a shallow cleft on the surface of the

catalytic domain. In contrast to this lies the cyclooxygenase active site deep within the catalytic domain and is accessible by a long, narrow channel (~ 5 x 25 Å) extending from the outer surface of the membrane binding domain to the centre of the catalytic domain. The catalytically crucial residue Tyr-385 is found at the apex of this channel. The residue Ser-530, which is acetylated by aspirin, lies just below Tyr-385, at a point where its acetylation can easily block access of the substrate to the active site. Other NSAIDs, like flurbiprophen also bind in this channel and thus seem to work by blocking access to the active site in a similar way as acetylation by aspirin. The structures of COX-1 and COX-2 are very similar and are superimposable, as can be expected from the high degree of amino acid identity. A crucial difference is, however, that the channel leading to the cyclooxygenase active site forks in the structure of COX-2, giving rise to an extra side pocket not observed in COX-1 (91-92). This unique feature in the structure of COX-2 is of particular importance for COX-2 specific inhibitors, the COXIBs.

Figure 6 shows the protein structures of COX-1 and COX-2 and indicates their composition of the three different domains as well as the active sites.

Biosynthesis of Prostanoids

Due to the chemical nature of its endoperoxide moiety, PGH_2 is very unstable in aqueous environment and decomposes rapidly to PGE_2, and PGD_2 with a half-life of about 5 minutes at 37°C (93-94). In living cells the reaction from PGH_2 to the biologically active prostanoids is enzymatically controlled in a cell-specific manner. Several PGH_2 isomerases and reductases leading to the five prostanoids are known. With the exception of the enzymes that form PGE_2, which will be discussed in more detail, the terminal synthases and their products will be shortly mentioned here.

Prostaglandin E_2 Synthases

Conversion of PGH_2 to PGE_2 involves the specific isomerisation of the endoperoxide moiety into a keto group at carbon 9 and a hydroxyl group at carbon 11 of the fatty acid chain. Three enzymes with PGE_2 synthase activity as their main activity have been described in the literature, one cytosolic and two membrane bound enzymes. Furthermore, two cytosolic anionic glutathione transferases of the μ class purified from human brain cortex are able to specifically form PGE_2 in the presence of GSH (95).

The cytosolic PGE_2 synthase (cPGES) is a member of the glutathione transferase family and requires GSH for its activity. cPGES has a molecular weight of 23 kDa and is identical to p23, a heat shock protein (Hsp) 90-binding protein. As such it was originally identified as a cofactor for the chaperone function of Hsp90 (96-98). Its PGE_2 synthase activity was not discovered until 2000 (98). The K_M and V_{max} values for recombinant cPGES expressed in *E. coli* are 14 μM and 190 nmol min^{-1} mg^{-1}, respectively. cPGES has been shown to be activated in the presence of ATP and Mg^{2+}, as well as in complex with Hsp90 (98).

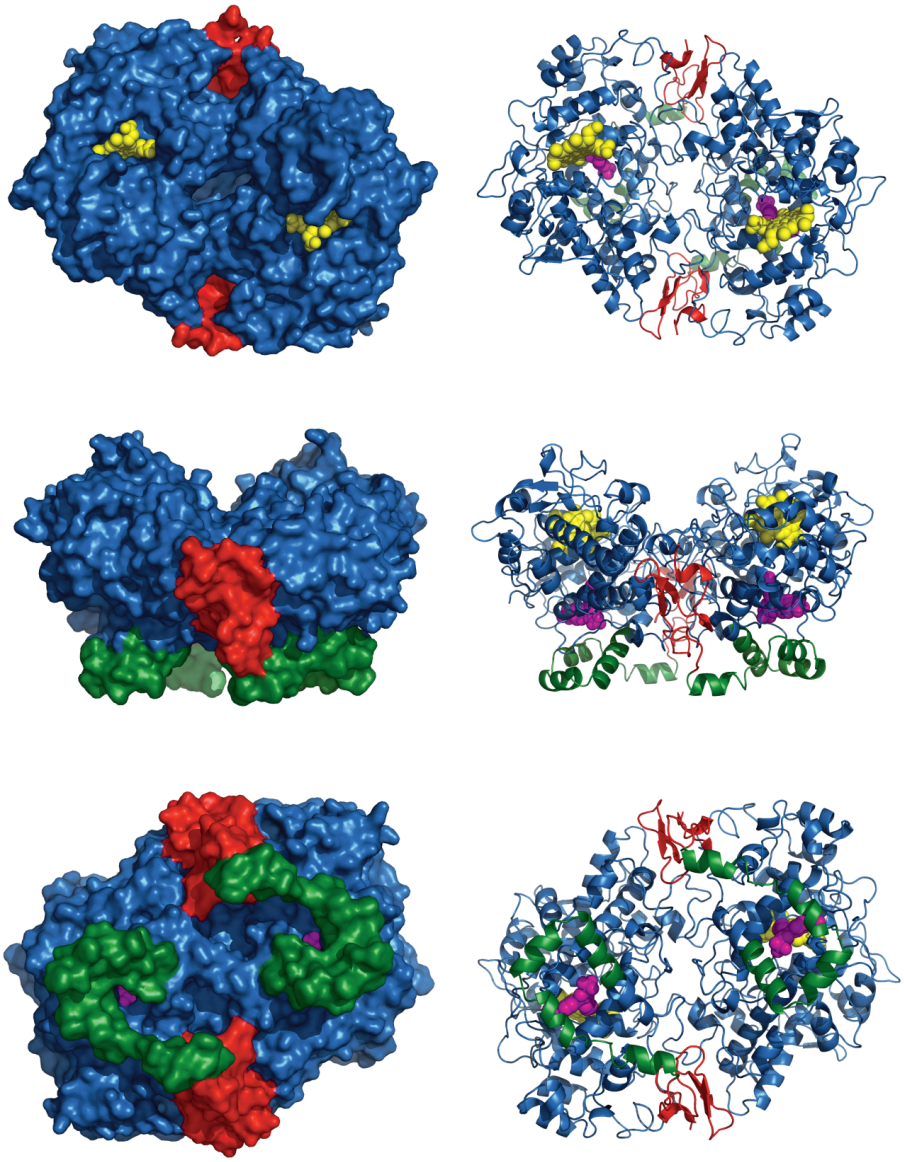


Figure 6. Crystal structures of ovine COX-1 and murine COX-2. *Both structures were solved by x-ray crystallography (PDB codes 1CQE and 6COX, respectively). COX-1 is depicted in surface representation (left) and COX-2 in cartoon representation (right). The N-terminal EGF-like domain is shown in red, the membrane binding domain in green and the catalytic domain in blue. The haem groups and inhibitors, which were co-crystallised with the proteins, are shown as black and magenta spheres, respectively. The top row represents the view from the lumen, the middle row a side view, and the bottom row shows the membrane face of the enzymes.*

cPGES is constitutively expressed in most cells and its expression levels are mostly unaltered by pro-inflammatory stimuli in various cells and tissues (98). However, an increase of activity has been observed in the cytosol of rat brain cells upon treatment with LPS. Furthermore, based on co-transfection experiments, a functional coupling between cPGES and COX-1 has been suggested (98). In these experiments co-transfection of cPGES with COX-1, but not with COX-2 in human embryonic kidney cells (HEK 293) resulted in a tenfold increase in PGE_2 formation as compared to control cells transfected with either COX isoform alone.

Mice deficient in the gene coding for cPGES have been developed (99). Pups from these animals die during the perinatal period and display retarded lung development reminiscent of the phenotype of neonates deficient in the glucocorticoid receptor complex. Analysis of AA metabolites in embryonic tissues and primary fibroblasts from these animals failed to support a function for cPGES in PGE_2 biosynthesis. Thus, although this enzyme can form PGE_2 *in vitro*, its role *in vivo* is associated with glucocorticoid receptor function and embryonic growth (99).

A GSH independent enzyme with PGE_2 synthase activity was identified in microsomes from rat (100) and cow (101-102). Monkey cDNA coding for this enzyme was cloned in 2002 (103), and named MPGES2.

Amino acids 1 to 88 of MPGES2, which form an initial N-terminal domain consisting of five rather hydrophobic α-helices, promote association with the membrane. These amino acids were found to be truncated in the initially identified bovine enzyme and later shown to be spontaneously cleaved off, leading to the formation of a mature MPGES2 protein that is distributed in the cytoplasm (104). The truncated MPGES2 is a homodimeric protein. Each monomer has a molecular weight of 33 kDa. The protein structure of monkey MPGES2 (PDB code 1Z9H) reveals that it is composed of three domains (105). The N-terminal domain consists of three α-helices and four β-strands. The central domain, which is mainly involved in dimerisation, consists of a large anti-parallel loop with a short helix at both ends, and the large domain at the C-terminus has an α-structure composed of eight helices connected by loops.

Recombinant MPGES2 has a broad specificity regarding reducing agents. It can utilise 2-mercaptoethanol, GSH, and dithiothreitol, in order of increasing effectiveness. Purified MPGES2 displays K_M and V_{max} values of 28 μM and 3.3 μmol min^{-1} mg^{-1}, respectively, with a pH optimum between 6 and 7 (103).

Northern blot analysis demonstrated that MPGES2 mRNA is mainly localised in the heart and in various regions of the brain, but, unlike MPGES1, not in the sexual organs.

MPGES2 knock-out mice have been recently developed (106), however, loss of MPGES2 expression did not result in a measurable decrease in PGE_2 levels in any tissue or cell type examined from these animals. Thus, neither cPGES (99) nor MPGES2 (106) seem to be essential for *in vivo* PGE_2 biosynthesis.

A third enzyme with pronounced PGE_2 synthase activity, MPGES1, was discovered in 1999 (30), just before cPGES and MPGES2. MPGES1 was initially referred to as microsomal glutathione transferase-1 like 1 (MGST1 L1) because of its high degree of

homology to the enzyme microsomal glutathione transferase-1 (MGST1) and, together with four more proteins, these two enzymes were allocated to the newly discovered protein superfamily of 'membrane associated proteins in eicosanoid and glutathione metabolism' (MAPEG), which is described in detail below.

MPGES1 is an inducible, GSH-dependent integral membrane protein with a molecular weight of 15-16 kDa. Human MPGES1 was recombinantly expressed in *E. coli* and displayed a K_M value of 160 µM and a V_{max} value of 170 µmol min^{-1} mg^{-1} at pH 7.4 (34).

Under normal physiological conditions expression of MPGES1 is low and found only in placenta, prostate, testis, mammary gland, and bladder, but not in most other organs (30). Like COX-2 however, MPGES1 expression is dramatically increased in various tissues by inflammatory stimuli like LPS, IL-1β, and TNF-α. The co-ordinated up-regulation of MPGES1 and COX-2 is reversed by the glucocorticoid dexamethasone (107-108) suggesting a functional coupling between MPGES1 and COX-2.

Besides the rapid conversion of PGH_2 to PGE_2, MPGES1 has also other catalytic activities. The close relationship of MPGES1 to MGST1 is revealed by its ability to slowly conjugate GSH to CDNB with a specific activity of 0.8 µmol min^{-1} mg^{-1}. Furthermore, MPGES1 catalyses the glutathione-dependent conversion of PGG_2 to 15-hydroperoxy-PGE_2 with a V_{max} value of 250 µmol min^{-1} mg^{-1}. This would provide an alternative pathway for PGE_2 production, since MPGES1 also possesses peroxidase activity and can reduce 15-hydroperoxy-PGE_2 to PGE_2 with a specific activity of 0.04 µmol min^{-1} mg^{-1}. However, the production of 15-hydroperoxy-PGE_2 exceeds its reduction by far (34).

Mice deficient in MPGES1 were generated by targeted homologous recombination on the inbred DBA/1lacJ background (37). These mice could not be distinguished from wt mice in their general behaviour, appearance, body weight, histology of several different tissues, or haematological parameters, indicating that MPGES1 does not play a role during development or in crucial physiological processes. However, when tested in different models of inflammation, including collagen induced arthritis and delayed type hypersensitivity, these mice displayed a marked reduction in inflammatory responses compared to their wt controls. Similarly, in the writhing test, a model of inflammatory pain induced by intraperitoneal injection of dilute acetic acid, these mice revealed a decrease in pain perception, which was comparable to the response that NSAID treated wt controls showed. Macrophages from MPGES1 deficient mice did not produce PGE_2 when stimulated with LPS, (109-110), and collagen antibody-induced arthritis, a model for human rheumatoid arthritis, was milder in MPGES1 knock-out mice than in wt mice (110). Furthermore, MPGES1 was demonstrated to play a role in neuropathic pain (111). Targeted deletion of the gene coding for MPGES1 showed thus a clear connection of this enzyme with inflammatory processes and inflammation related pain perception.

Taken together, out of the three enzymes with PGE_2 synthase activity that have been identified to date cPGES and MPGES2 have been shown to be constitutively expressed and to be preferentially coupled to COX-1, while MPGES1 has been demonstrated to be up-regulated by pro-inflammatory stimuli in concert with COX-2 and to be involved in pathophysiologic PGE_2 formation during inflammatory reactions.

Prostaglandin D_2 Synthases

The reaction that forms PGD_2 from PGH_2 is also an isomerisation reaction, but in contrast to the formation of PGE_2, the keto group and the hydroxyl group end up in the opposite position, i.e. at carbon 11 and carbon 9, respectively. To date there are two known enzymes that catalyse this reaction, Lipocalin-type PGD Synthase (L-PGDS) and Haematopoietic PGD Synthase (H-PGDS).

L-PGDS, previously known as brain-type PGD Synthase, is a 26 kDa secretory protein that is mainly expressed in the central nervous system and by epithelial cells of the epididymis as well as by Leydig cells in the testis, and it is secreted into the cerebrospinal fluid and the seminal plasma (112). H-PGDS, on the other hand, belongs to the family of cytosolic glutathione transferases, forming a class of its own, the σ class. H-PGDS is responsible for the biosynthesis of PGD_2 in immune and inflammatory cells, among others in mast cells, antigen presenting cells, and T cells (112).

As one of its major physiological roles PGD_2 is involved in the promotion of sleep (113), and it reduces the body temperature during the sleep phase. In this function it acts opposite to PGE_2. PGD_2 is furthermore involved in asthmatic diseases and causes contraction of the bronchial airways.

Prostaglandin F_2 Synthases

Only two out of four possible stereoisomers of PGF_2 are formed *in vivo*. $PGF_{2\alpha}$ can be obtained from a reduction of the endoperoxide moiety in PGH_2 as well as from a reduction of the keto group at carbon 9 of PGE_2. The two hydroxyl groups of the product are in both cases on the same side of the cyclopentane ring, i.e. in α-position. When PGD_2 is reduced at its keto group at carbon 11, 9α-,11β PGF_2 is formed instead.

The enzyme that catalyses $PGF_{2\alpha}$ from PGH_2, 9,11-endoperoxide reductase, constitutes a membrane-associated, monomeric, GSH dependent enzyme with an apparent molecular weight of 17 kDa. 9,11-endoperoxide reductase belongs to the glutathione transferase family, however, it cannot use 1-chloro-2,4-dinitrobenzene (CDNB), a substance which is commonly used as electrophilic substrate for glutathione transferases in order to determine glutathione transferase activity in standardised laboratory assays (114).

PGE 9-ketoreductase catalyses the reduction of PGE_2 to $PGF_{2\alpha}$. It constitutes a 37 kDa cytosolic enzyme with a catalytic activity that is dependent on NADH or NADPH as a co-factor. Based on a high degree of amino acid similarity as well as similar substrate specificities PGE 9-ketoreductase was grouped to be a member of the aldo-keto reductase superfamily (114).

PGD_2 can be reduced to yield $PGF_{2\alpha}$ by the action of PGD 11-ketoreductase, a cytosolic protein with an apparent molecular weight of 37 kDa. Similar to PGE 9-ketoreductase it is dependent on NADH or NADPH as co-factor, and it also displays a high homology to proteins from the aldo-keto reductase superfamily. In contrast to the

PGE_2 reducing enzyme, however, PGD 11-ketoreductase is able to also catalyse the reduction of the endoperoxide moiety in PGH_2 in addition to its reductase activity (114).

$PGF_{2\alpha}$ production is found in various organs and leads to the contraction of smooth muscle cells. Because notable amounts of $PGF_{2\alpha}$ are found in the uterus and the corpus luteum in response to hormonal stimulation, it is believed to play a particular role in reproduction (115).

Thromboxane A_2 Synthase

Thromboxane A_2 Synthase (TXAS) activity was first described in platelets in 1974 (18, 20). Two years later the enzyme that catalyses the conversion of PGH_2 to TXA_2 was identified (116) and subsequently found to belong to the CYP P450 family (117). However, this relationship was based on spectroscopic characteristics as well as sequence similarities (118) rather than on functional properties. TXAS was characterised to be a 59 kDa membrane bound protein with an enzymatic activity dependent on haem as a co-factor (119). High amounts of TXAS are expressed in platelets and in macrophages.

TXA_2 is produced by activated platelets and shows pro-thrombotic properties (18). It stimulates the activation of new platelets and increases platelet aggregation. By activating its specific receptor on vascular smooth muscle cells TXA_2 has furthermore effects as a vasoconstrictor. These functions make it a central player in the physiological process of haemostasis, but also in pathophysiological conditions such as cardiovascular disease and stroke. In platelets TXAS is functionally coupled to COX-1, and administration of low doses of aspirin, which preferentially inhibits COX-1, has a protective effect against acute myocardial infarction (120-122).

TXA_2 has a very short half-life of approximately 30 seconds in aqueous solution at 37°C (116) and is non-enzymatically hydrolysed to yield TXB_2, which is a more stable, but biologically inactive metabolite of TXA_2 that can be detected in biological samples (123). The degradation is temperature-dependent, resulting in a half live of about 1 minute at 22°C, and about 10 minutes at 0°C (116). Further metabolism of TXB_2 yields 11-dehydro TXB_2, 11-dehydro-13,14-dihydro-15-keto TXB_2, and 2,3-dinor TXB_2, which reflect the systemic levels of TXA_2 in plasma (124).

Prostacyclin Synthase

An enzymatic activity transforming PGH_2 to an unstable metabolite that could inhibit platelet aggregation was discovered in microsomes from rabbit and pig aorta shortly after the discovery of TXA_2 and its pro-thrombotic properties (25). It was located in endothelial cells as well as in smooth muscle cells and allocated to a CYP P450 enzyme (125). After initial purification attempts using classical fractionation techniques failed, Prostacyclin Synthase (PGIS) was eventually purified by immunoaffinity chromatography using specific monoclonal antibodies (126). PGIS is, like TXAS, a membrane bound haemoprotein with a molecular weight of 52 kDa.

PGI_2 acts in many ways as an opponent to TXA_2 by having effective vasodilator function and antithrombogenic properties. PGI_2 inhibits platelet activation and thus prevents thrombus formation during primary haemostasis (25). Furthermore, it can disrupt existing thrombi of previously aggregated platelets (127). Therefore, PGI_2 is generally regarded as an important, beneficial homeostatic regulator of the cardiovascular system.

Also regarding its stability, PGI_2 is similar to TXA_2. It is rapidly degraded in aqueous solution by non-enzymatic hydrolysis to yield 6-keto $PGF_{1\alpha}$. The anti-aggregatory activity of PGI_2 is lost within 10 min at 37°C and within 20 min at 22°C (128-129). 6-keto $PGF_{1\alpha}$ is further metabolised, and at least 16 compounds could be detected in urine from human volunteers that were injected with radiolabelled PGI_2 (130). 2,3-dinor-6-keto $PGF_{1\alpha}$ was found to be the major urinary metabolite in man (131-133).

Prostaglandin E_2

Receptors for Prostaglandin E_2

PGE_2 mediates a number of biological responses through four subtypes of prostaglandin receptors, named EP_1 to EP_4, which all belong to a distinct subfamily of the GPCR superfamily (134-135). These GPCRs are expressed on the surface of different target cells. For instance, EP_2 and EP_4 can be found on ovarian cells and osteoclasts, where they participate in maturation and ovulation, and in bone resorption, respectively, while EP_1 and EP_3 are expressed by neurons and cause fever and pain responses when activated.

Receptors EP_2 and EP_4 signal through a G_s-mediated stimulation of the adenylate cyclase and increase the intracellular cyclic adenosine monophosphate (cAMP) concentration of the target cells. EP_3 is regarded as an inhibitory receptor that couples to G_i-proteins and decreases the formation of cAMP. The EP_1 receptor signals through a G_q-mediated increase in intracellular DAG, IP_3 and Ca^{2+}.

Catabolism of Prostaglandin E_2

The operating distance of PGE_2 is, as for all primary prostanoids, very limited. It usually acts in an autocrine or paracrine manner only on the same cell of its synthesis or on immediately adjacent cells and is rapidly degraded shortly after its formation with an *in vivo* half-life of less than one minute (136). The first step in the catabolism of PGE_2 is the oxidation of the hydroxyl group at carbon 15 to a ketone, catalysed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (137-138). This is followed by the reduction of the Δ^{13} double bond by 15-ketoprostaglandin Δ^{13}-reductase (13-PGR). The resulting metabolite, 13,14-dihydro-15-keto PGE_2, accumulates to detectable levels in plasma (139) but has significantly reduced activity (140). 13,14-dihydro-15-keto PGE_2 degrades non-enzymatically in plasma at 37°C with a half-life of about 45 minutes (141), while its *in vivo* half-life was found to be only about 8 minutes (136). *In vivo* it is further metabolised via one or two steps of β-oxidation from the carboxyl end to yield dinor or tetranor compounds, and via ω-oxidation to yield ω1 and ω2 hydroxy compounds and

eventually dicarboxylic acids, which are excreted into the urine (142). The major metabolite of PGE_2 found in the urine of humans is 13,14-dihydro-15-keto-2,3,4,5-tetranor-prostan-1,20-dioic acid (tetranor-PGEM) (143).

The enzymes involved in the metabolism of PGE_2 are ubiquitously expressed in mammalian tissues (144). Both 15-PGDH and 13-PGR are cytosolic enzymes. 15-PGDH has a molecular weight of 29 kDa, is functionally active as a homodimer and is dependent on NAD^+ as a co-factor (145), while 13-PGR is a 56 kDa enzyme with an activity that is dependent on either NADH or NADPH as an electron donor (146-147). The enzymatic machinery that carries out β-oxidation, consisting of medium-chain specific acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, acetyl-CoA acyltransferase, and electron transfer flavoprotein-ubiquinone oxidoreductase are expressed in the mitochondrial matrix or the mitochondrial inner membrane, respectively (148), and ω-oxidation is achieved by CYP P-450 enzymes that have a wide tissue distribution including prostate and seminal vesicles, kidney, liver, stomach and small intestine (149-151).

Although this catabolic pathway is believed to account for most of the PGE_2 metabolism, there are different minor pathways as well. For instance PGE_2 can be reduced by PGE 9-ketoreductase to form $PGE_{2\alpha}$, as described above.

Pharmacological Inhibition of Prostanoid Formation

Due to the importance of prostanoid signalling in many pathophysiological conditions the biosynthetic pathway leading to prostanoids is the target for numerous pharmaceuticals. These pharmaceuticals are used in the therapy of inflammatory diseases, pain, and fever, as well as in the prevention of cardiovascular events. The most commonly consumed class of these pharmaceuticals are the non-steroidal anti-inflammatory drugs, or NSAIDs, small molecule inhibitors of the catalytic activity of COX. The term 'non-steroidal' is used to distinguish NSAIDs from corticosteroid drugs, another class of pharmaceuticals involved in the regulation of inflammation. Natural and synthetic corticosteroids employ, however, a different mechanism of action. They influence the expression of key enzymes in the generation of PGE_2, including $cPLA_2$-α (46), COX-2 and MPGES1 (107-108). Thus, corticosteroids lead to the inhibition of prostanoid formation and exert similar anti-inflammatory effects as NASIDs. It should be noted, however, that besides this, corticosteroids elicit also a broad range of other effects.

Direct inhibition of the downstream enzyme MPGES1 has emerged as a novel strategy in the development of anti-inflammatory drugs. In contrast to the available treatments, MPGES1 inhibition is supposed to act in a more specific way and hence display less severe side effects.

Traditional NSAIDs and COX-2 Specific Inhibitors

NSAIDs belong to the most widespread pharmaceuticals with a long history of application. Since the early 1970s, it has been known that NSAIDs interfere with prostanoid formation, and COX was identified as their target enzyme (22). After the discovery of the second, inducible COX isoform, COX-2 (85-86), and after it became clear that this isoform was largely responsible for prostanoid formation associated with inflammation, while the housekeeping enzyme COX-1 was found to be responsible for physiological functions, the pharmacological industry immediately launched screening programmes to identify candidate drugs with selectivity for COX-2. The first drugs that resulted from these screening programmes were celecoxib (152-153) and rofecoxib (154-155). Thus, the class of non-steroidal anti-inflammatory drugs split into two groups, a first generation of NSAIDS, i.e. the traditional NSAIDs, and a second generation, the COXIBs. COXIBs inhibit specifically the activity of COX-2, ideally without impairment of COX-1, while traditional NSAIDs are unselective inhibitors of both isoforms of COX.

Traditional NSAIDs and COXIBs vary in their spectrum of both desired and undesired drug effects because of different expression patterns and coupling to different downstream enzymes of the two COX isozymes affected by these drugs. However, it should be noted that the degree of selectivity that different representatives of the two

groups display should be considered as a continuous variable, so that COX-2 specific inhibitors, at a sufficiently high concentration, might also affect COX-1 (156).

Several traditional NSAIDs are available, and most of them are derivatives of different acids, including salicylic acid (aspirin), propionic acid (e.g. ibuprofen, naproxen, flurbiprofen), acetic acid (e.g. indometacin, sulindac, diclofenac), fenamic acid (e.g. mefenamic acid, flufenamic acid), and enolic acid (e.g. piroxicam, meloxicam). Traditional NSAIDs block the cyclooxygenase activity of COX but leave the peroxidase activity unaffected. This can be explained by their mode of action. Several NSAIDs have been shown to bind within the hydrophobic channel that leads to the cyclooxygenase active site and thus exclude the substrate AA from access (91). The peroxidase active site is unaffected from inhibitor binding.

Most traditional NSAIDs are competitive inhibitors that reversibly bind and compete with the substrate for the same binding site. A notable exception is aspirin, which also rapidly binds in a reversible manner; however, when acting on COX-1, it subsequently reacts with Ser-530 of the enzyme and covalently modifies this residue, resulting in an irreversible inhibition (157). The corresponding residue in COX-2 that is acetylated by aspirin is Ser-516 (158-159).

Traditional NSAIDs prevent the formation of PGH_2 from both COX isozymes and hence also the subsequent formation of all primary prostanoids. This can lead to severe adverse drug effects because of the many physiological effects that prostanoids display. Among the most common side effects connected with traditional NSAIDs are gastrointestinal effects (160). It is believed that basal levels of PGE_2 are necessary to maintain homeostasis of the gastric mucosa. Several mechanisms are presumably involved in the gastroprotective role of PGE_2, including maintenance of the mucous bicarbonate barrier and induction of growth factors such as vascular endothelial growth factor (161). Traditional NSAIDs eliminate these basal levels of PGE_2, leading to gastro-duodenal ulcers in about 15-30%, and even to life-threatening complications in up to 2% of patients taking these drugs (161).

One of the main goals with the development of COXIBs was the prevention of these adverse drug effects. The so called 'COX-2 hypothesis' attributed the gastrointestinal intolerance entirely to inhibition of COX-1 derived prostanoids, while induced PGE_2 formation during inflammatory reactions was entirely attributed to the function of COX-2. This was in agreement with the discovery of MPGES1 (30) and its co-regulation with COX-2 by pro-inflammatory cytokines (107-108), which was found shortly after the first COXIBs were developed. It was believed that COXIBs prevented inflammation and pain without impairing the physiological functions that COX-1 derived prostanoids achieve.

The first COXIB to be developed was celecoxib, which is marketed under the brand name Celebrex or Celebra. Using a whole blood assay, it shows a moderate, 8- to 30-fold selectivity for COX-2 compared to COX-1 (162-163). The second COXIB that entered the market, rofecoxib, which is also known under its brand name Vioxx, is a highly selective and potent COX-2 inhibitor compared to celecoxib. Rofecoxib showed a 35- to 272-fold selectivity for COX-2 in the whole blood assay (162-163). Several other

COXIBs with improved COX-2 selectivity were developed after these two drugs, including valdecoxib (brand name Bextra), parecoxib, which is a water soluble and injectable prodrug of valdecoxib, etoricoxib (brand name Arcoxia), and lumiracoxib (brand name Prexige).

All of these drugs are more selective for COX-2 than for COX-1 because the substrate binding site, which is occupied by the inhibitors, differs between the two enzymes. Changes in the hydrophobic channel leading to the cyclooxygenase active site have occurred. While several small amino acids (Val-434, Arg-513, and Val-532) are found in the substrate tunnel of COX-2, the corresponding positions in COX-1 incorporate larger amino acids (Ile-434, His-513, and Ile-532). This makes the tunnel and thus also the substrate or inhibitor binding site of COX-2 wider and creates an extra cavity, allowing larger molecules to bind (90-92).

Initial, short-termed studies that compared the effects of traditional NSAIDs and COXIBs showed a marked superiority of COXIBs, with reduced signs of inflammation while the number of adverse gastrointestinal events was significantly reduced (164-165). The same result was reported as the outcome of two large trials, the Vioxx gastrointestinal outcome research (VIGOR) study (166), and the Celecoxib long-term arthritis safety study (CLASS) (167). Both studies concluded that the tested COXIBs were associated with significantly fewer adverse gastrointestinal effects. The latter study also reported no significant difference in incidence of cardiovascular events in patients not taking aspirin. However, it turned out that in particular the CLASS trial, which was financed by Pharmacia, the manufacturer of celecoxib, presented the data only partially. Analysis of the complete information available to the United States Food and Drug Administration contradicted the published conclusions and showed similar numbers of gastrointestinal complications in the comparison groups. Almost all the gastrointestinal complications that had occurred during the second half of the trials were related to celecoxib (168). Subsequent studies showed that the degree of selectivity of different NSAIDs for one particular COX isozyme correlated with characteristic effects. In general, drugs that mainly inhibit COX-1 display a high gastrointestinal risk, while highly COX-2 specific drugs are associated with a small but absolute risk to develop cardiovascular complications. The adenomatous polyp prevention on Vioxx (APPROVe) study found that the use of rofecoxib was associated with an increased cardiovascular risk (169), which led to the worldwide withdrawal of Vioxx on September 30, 2004. Bextra with its active agent valdecoxib was then withdrawn from the market in the United States, Australia and Europe, while celecoxib is still available. However, the number of prescriptions for celecoxib declined substantially (156).

Mechanistic studies revealed the reason for the cardiovascular hazard associated with COXIBs. Both celecoxib and rofecoxib reduce 2,3-dinor $PGF_{1\alpha}$, the urinary metabolite of PGI_2, to a similar extend as the traditional NSAIDs ibuprofen or indomethacin. However, while the traditional NSAIDs also decrease 11-dehydro TXB_2, the major urinary metabolite of TXA_2, COXIBs have no such effect (170-171). This is in agreement with findings that celecoxib does not inhibit TXA_2 dependent platelet aggregation *ex vivo* (170) and that COX-2 is not expressed in mature platelets and hence not involved in

TXA_2 biosynthesis (172). Both COX-1 and TXAS are, on the other hand, expressed by platelets (18, 20), and the anti-coagulative effect of aspirin, which, at low doses, inhibits almost only COX-1, is well known (120-122). PGI_2 is mainly formed by endothelial cells (173), and even though this cell type expresses both COX-1 and COX-2 in addition to PGIS, it appears to be largely derived from COX-2 (156).

Thus, the use of COXIBs is accompanied with an increased incidence of severe cardiovascular side effects leading to myocardial infarction and stroke because selective inhibition of COX-2 in PGI_2 producing endothelial cells removes a protective constraint on blood coagulation, while the COX-1 dependent production of pro-coagulative TXA_2 in platelets is unaffected.

Microsomal Prostaglandin E Synthase-1 Inhibitors

'A novel target for drug development in the areas of inflammation and possibly cancer' (30) was identified with the discovery of MPGES1. MPGES1 is functionally coupled to COX-2 (107-108) and was shown to play a pivotal role in PGE_2 formation during inflammatory reactions. Its role in inflammatory diseases was demonstrated in several models of inflammation using mice deficient in MPGES1 (37). Furthermore, MPGES1 acts downstream of COX, so it is expected that inhibition of this enzyme will result in the elimination of only induced PGE_2 under pro-inflammatory conditions, while basal physiological levels of PGE_2 produced by cPGES or MPGES2 are not affected by specific MPGES1 inhibitors.

When it became clear that COXIBs are associated with infrequent but severe undesired drug effects due to accelerated thrombogenesis, but neither thrombogenesis nor blood pressure was affected in MPGES1 knock-out mice, and moreover, PGI_2 was found to be increased in these mice while levels of PGE_2 were decreased (174), the strategy of MPGES1 inhibition for the treatment of inflammatory diseases with a better ratio of desired to undesired drug effects was strengthened.

Several molecules have been shown to interfere with MPGES1 function, employing different mechanisms. Among those are unselective and selective inhibitors of the catalytic activity of MPGES1, inhibitors that selectively interfere with the up-regulation of MPGES1, as well as selective inhibitors of the catalytic activity of both MPGES1 and 5-LO, leading to a depression of both PGE_2 and leukotrienes (175). For the work included in this thesis selective inhibitors of MPGES1 activity are of highest interest. Albeit most of the substances that are described in the literature to date do not yet fulfil the criteria that need to be met for a safe drug to be used in humans, initial *in vivo* results from animal experiments are being published (176-179).

The MAPEG Protein Superfamily

The acronym MAPEG denotes a protein superfamily of 'membrane associated proteins that are involved in eicosanoid and glutathione metabolism'. Today it is known that the members of this protein superfamily are integral membrane proteins, i.e. they are permanently attached to the membrane by spanning it with several transmembrane domains instead of being only loosely associated to biological membranes, as the name might imply.

Functionally, this superfamily can be divided into two groups, the first of which comprises proteins involved in the endogenous metabolism of physiologically important reactive oxygenated lipid mediators, while the second group comprises proteins that are involved in detoxification of highly reactive lipophilic compounds of both exogenous and endogenous origin (31). Hence, most of the MAPEG members are enzymes that display either glutathione transferase activity or glutathione dependent peroxidase or isomerase activity. MAPEG members are found in prokaryotes and eukaryotes, but not in archaeal organisms. A total of 136 MAPEG proteins have been identified in database screens so far (180).

Despite the diverse biological functions that the members of the MAPEG family carry out, all of these proteins demonstrate very similar biochemical characteristics, such as a consistent length of about 150 amino acids. They also share certain structural features like a hydropathic profile containing four hydrophobic regions, which seem to correspond to four transmembrane domains. Typical members of the MAPEG superfamily are basic proteins with an isoelectric point between 10 and 11, they have several conserved sequence motifs, and similar enzymatic activities depending on their particular subgroups.

The six mammalian members of the MAPEG superfamily are the 5-lipoxygenase activating protein (FLAP), leukotriene C_4 synthase (LTC4S), microsomal glutathione transferase-1 (MGST1), microsomal glutathione transferase-2 (MGST2), and microsomal glutathione transferase-3 (MGST3), as well as microsomal prostaglandin E synthase-1 (MPGES1). These mammalian proteins are by far the most investigated members of the superfamily, so the detailed description below will therefore concentrate on them. MPGES1, the enzyme in the centre of interest of this thesis, has already been described together with the enzymes capable of catalysing PGE_2 from PGH_2.

Based on multiple sequence alignments and their evolutionary relationships, proteins of the MAPEG superfamily can be classified into eight eukaryotic and three prokaryotic families. For the eukaryotic MAPEG members, each of the six mammalian proteins mentioned above forms its own family. These six protein families are joined by the family of insect MAPEG proteins and the family of MAPEG proteins from waterliving organisms. The prokaryotic families are denoted *E. coli* MGST cluster, *Synechocystis* MGST cluster and remaining bacteria.

Because most research focuses on the mammalian members of the MAPEG superfamily, a more general subdivision into four major classes has been made, which combines the closely related protein families involved in leukotriene biosynthesis (i.e., LTC4S, FLAP and MGST2) with the family of aquatic organisms in the first group. The MGST3 family forms a second group together with several related plant and fungal proteins, and all prokaryotic proteins make up the third group. The fourth and last group contains the closely related proteins MGST1 and MPGES1 along with the insect family of MAPEG members (Figure 7) (32, 180).

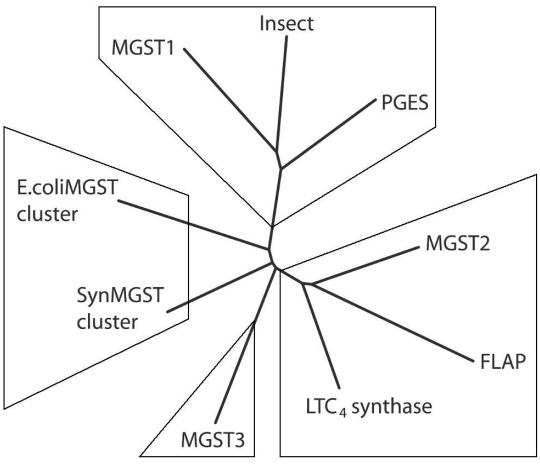


Figure 7. Major subgrouping of the MAPEG superfamily. *A schematic evolutionary tree based on sequence similarities shows the relationship between the different groups of the MAPEG superfamily. Figure modified from reference (180).*

Structural Features of the MAPEG Protein Superfamily

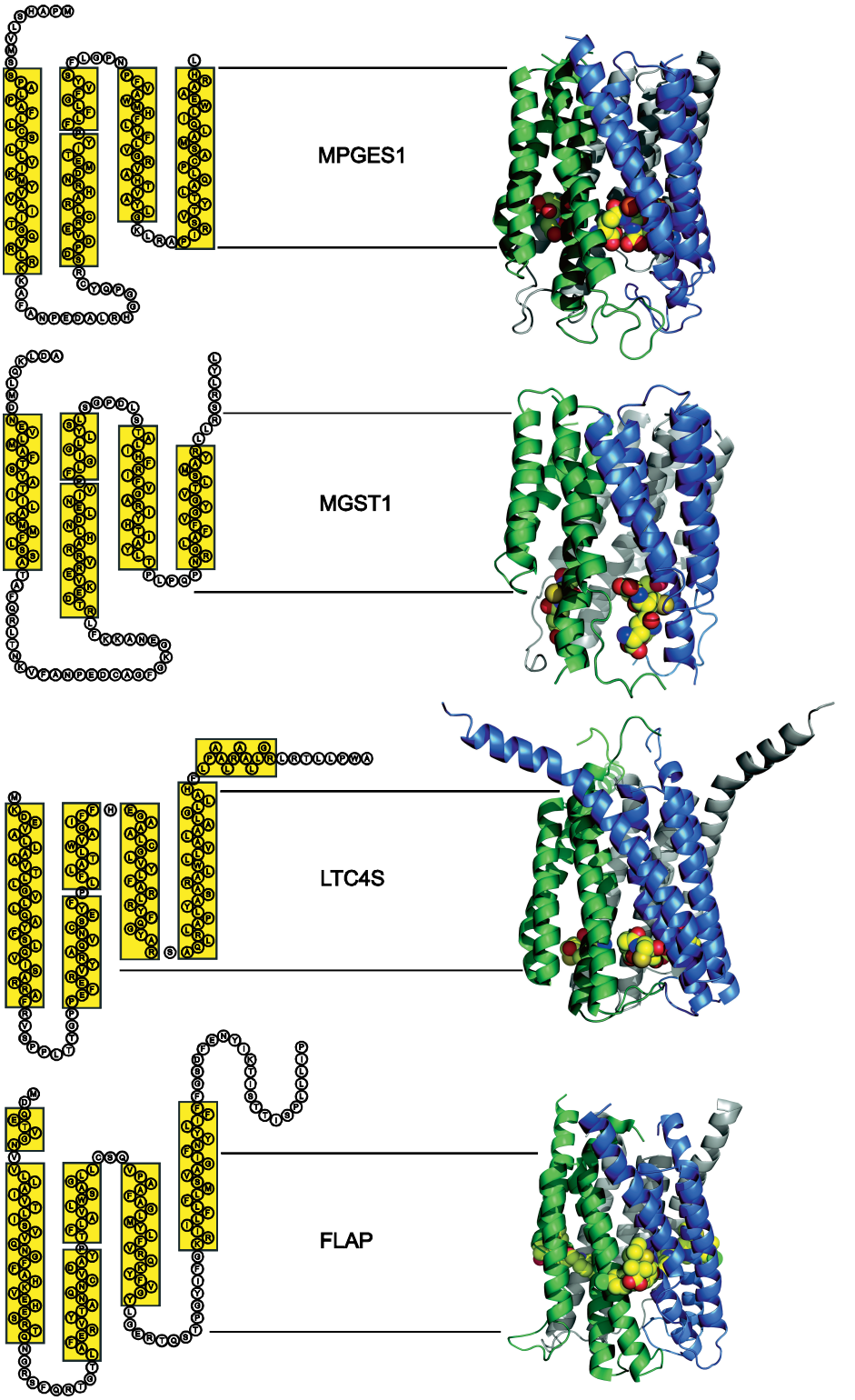
Structural information of a protein is of great importance for the understanding of cellular and molecular processes on an atomic level. Especially for enzymes, the protein structure can provide valuable insights into the reaction mechanism or the impact of mutations. The structure of a protein might furthermore be of great help for the tailored development of pharmaceuticals. Because of the important enzymatic function of most of the MAPEG members and their medical relevance their structural elucidation is in the limelight of interest.

The structures of four out of the six mammalian MAPEG members have been solved during the recent years. The first MAPEG structure to be solved was the structure of rat MGST1 (PDB code 2H8A). It has been solved in 2006 in complex with glutathione to a resolution of 3.2 Å (181). Electron crystallography employing two dimensional crystals of purified protein that was reconstituted into a phospholipid bilayer was used for structural elucidation of MGST1. This structure was followed one year later by the structures of FLAP and LTC4S, which were published virtually at the same time in 2007. Both proteins were studied by x-ray crystallography using three dimensional crystals of detergent solubilised protein. The structure of FLAP was solved in complex with two different leukotriene biosynthesis inhibitors; together with MK-591 (PDB code 2Q7M) to a resolution of 4.0 Å, and together with an iodinated analogue of MK-591 (PDB code 2Q7R) to a resolution of 4.2 Å. FLAP is to date the only member of the MAPEG

superfamily on which structural information about the inhibitor binding site is available. One week after publication of the FLAP structure, the structure of LTC4S was published independently by two different groups. While the first group (182) reached a resolution of 3.3 Å for LTC4S in complex with glutathione (PDB code 2PNO), the second group (183) managed to elucidate the structure of LTC4S in its apo form to a resolution of 2.0 Å (PDB code 2UUI) and in complex with glutathione to a resolution of 2.15 Å (PDB code 2UUH). Recently, yet another structure of LTC4S has been deposited in the PDB. This structure (PDB code 3HKK) shows the protein at a resolution of 2.9 Å in complex with glutathione sulfonate, an analogue of glutathione, which is known to inhibit the activity of many MAPEG members because the crucial reactive group of the cysteine moiety is modified in glutathione sulfonate. Finally, in 2008 the structure of MPGES1 (PDB ID code 3DWW) was solved to a resolution of 3.5 Å using electron crystallography of two dimensional protein crystals (184). The publication describing this structure is included as paper I in this thesis.

In agreement with the high degree of sequence homology between the proteins of the MAPEG superfamily, a common fold seems to be adopted by its members. This is supported by all structures obtained to date (Figure 8). All MAPEG members adopt a trimeric arrangement of identical subunits. Heterotrimers of subunits coming from different MAPEG members have not been observed, and judged by diverse polar intersubunit contacts of different MAPEG members they are also unlikely to form (185). A single subunit consists of a bundle of four α-helices that transverse the membrane. Both the N-terminus and the C-terminus of one protein are located on the same side of the membrane. The transmembrane helices are connected to each other by poorly structurally defined loops. The loop between TM2 and TM3, which is located on the same side of the membrane as the N- and the C-terminus, is usually very short, consisting only of a few amino acid residues. The other two loops, which connect TM1 and TM2 and TM3 and TM4, respectively, are substantially longer, although the actual length of these loops varies considerably between the individual proteins. Compared with the N- and the C-terminus they are located on the opposite side of the membrane. Many positively charged residues are located within these two loops. This leads to the assumption that they are located *in vivo* on the cytosolic side of the membrane (186), which would place the N- and the C-terminus on the luminal side of the endoplasmic reticulum. However, the actual membrane topology remains to be proven.

Figure 8. Structural features of the MAPEG superfamily. *The structures of four MAPEG members have been determined. They display a similar membrane topology (left) and a common overall structure (right). The subunits of the homotrimeric proteins are depicted in green, blue and grey, respectively. MPGES1, MGST1, and LTC4S bind GSH as a co-factor, whereas FLAP was co-crystallised with the inhibitor MK-591, which binds at the same site as GSH. GSH and MK-591 are shown as spheres with carbon atoms coloured in yellow. The membrane boundaries are indicated by horizontal lines. The luminal side of the membrane is on the top and the cytosolic side on the bottom.*


MPGES1
MGST1
LTC4S
FLAP

Within one homotrimer, the four transmembrane helices of each subunit are arranged relative to each other in a very characteristic way, in which the three TM2s come together to form an inner core of the trimer. The other three transmembrane helices of each subunit are grouped around this inner core, so that TM1 of one subunit makes contact with TM4 of an adjacent subunit (Figure 9). GSH is bound at this interface between the subunits. Residues from all helices form the GSH binding site. Especially, a common signature motif in the highly conserved TM2 consisting of the residues RXXXNXXE/D is suggested to be involved in the binding of GSH (187). FLAP is the only exception, in which these residues have been replaced, which is consistent with the fact that FLAP does not bind GSH and lacks enzymatic activity. In LTC4S and MPGES1, GSH was found to bind to the protein in a U-shaped conformation (182-184), whereas it adopted an elongated conformation in MGST1 (181). It remains to be shown whether this different conformation of GSH in MGST1 can be confirmed and, if it is correct, whether it is connected to the particular function of this enzyme.

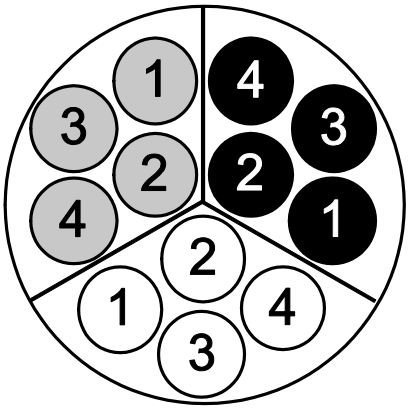


Figure 9. Homotrimeric organisation of MAPEG members. *An inner core, formed by the three TM2s from each subunit, is present in all known MAPEG structures. The remaining helices are grouped around this core, so that TM1 of one subunit makes contact to TM4 of an adjacent subunit. The putative active site or inhibitor binding site, respectively, is located at the subunit interface.*

5-Lipoxygenase Activating Protein

FLAP was first described in 1990 as an 18 kDa protein targeted by the leukotriene biosynthesis inhibitor MK-886, to which it binds with high affinity (188-189). The newly discovered protein was named 5-lipoxygenase activating protein because transfection studies in human osteosarcoma cells indicated that calcium ionophore induced leukotriene production only occurred when both 5-LO and FLAP were present. Furthermore, changes in FLAP expression greatly influence the cellular leukotriene synthetic capacity.

Although expression of FLAP is crucial for leukotriene synthesis, no intrinsic enzymatic activity has been found. This feature clearly separates FLAP from all other members of the MAPEG superfamily and substantiates the fact that FLAP is the only MAPEG member that does not bind GSH as essential co-factor (190). FLAP is located in the membranes of the nuclear envelope, because of which a possible role as a docking

protein for 5-LO was suggested. However, there is no evidence for direct interaction between FLAP and 5-LO, and MK-886 can inhibit leukotriene production without any effect on the translocation of 5-LO (33, 59). FLAP is able to specifically bind AA and is assumed to transfer it to 5-LO, and the binding site of FLAP is competitively inhibited by leukotriene synthesis inhibitors (191). Furthermore, FLAP is required for efficient utilisation of 12(S)-HETE and 15(S)-HETE as a substrate for 5-LO. Thus, it is believed that FLAP acts as a general lipid carrier protein and a substrate supplier for 5-LO (192).

Leukotriene C_4 Synthase

LTC4S catalyses a glutathione transferase reaction, in which GSH is conjugated to the leukotriene precursor LTA_4 to form LTC_4. However, it differs from other glutathione transferases by its failure to conjugate GSH to xenobiotics and by its substrate selectivity for LTA_4 and analogues.

LTC4S was purified in 1993 (35) and characterised as a membrane protein consisting of 150 amino acid residues with a molecular weight of 18 kDa. The K_M and V_{max} values for LTA_4 are 3.6 µM and 1.3 µmol min^{-1} mg^{-1}, respectively. The enzyme activity is augmented by Mg^{2+} and inhibited by Co^{2+}, NEM and the indole MK-886 (193).

LTC4S activity has been described in eosinophils, basophils, mast cells, and certain phagocytic mononuclear cells. Also, human endothelial cells (194), vascular smooth muscle cells (195), and platelets all express LTC4S activity without concomitant expression of 5-lipoxygenase. The formation of LTC_4 in these cells is therefore dependent on the transcellular metabolism of LTA_4, for instance by interaction with activated neutrophils (194-199).

Based on data from gel filtration chromatography (35), as well as from bioluminescence resonance energy transfer studies (36), it was initially believed that LTC4S is enzymatically active as a homodimer. However, calculation of the projection map of LTC4S at a resolution of 4.5 Å using data obtained from electron crystallography revealed a homotrimeric organisation of the enzyme (200). This homotrimeric quarternary structure was eventually proven in 2007, when the structure of LTC4S was elucidated (182-183). The structural insights allowed for speculations about the active site and the reaction mechanism of LTC4S (183). Consitent with MGST1 and MPGES1, GSH is bound at the interface of two adjacent monomers close to the membrane face, indicating the location of the active site. GSH is bent in LTC4S to adopt a U-shaped conformation, directing its thiol group towards the membrane interface where it interacts with Arg-104. Furthermore, a detergent molecule was found to bind in the hydrophobic cleft between the subunits. This detergent molecule has important structural similarities to LTA_4 regarding its amphipatic nature and its overall length and thus serves as a model for the substrate. The ω-end of the detergent molecule is positioned by residue Trp-116, which serves as a molecular ruler. If LTA_4 is modelled into this position in the putative active

site, carbon 6 and the epoxide of LTA_4 are positioned near the reactive cysteinyl thiol of GSH. The suggested reaction mechanism predicts that binding of GSH induces slight conformational changes compared to the apo form of the enzyme and enables the lipophilic substrate LTA_4 to enter the active site from the membrane. Arg-104 is predicted to act as catalytic residue and activate GSH to its anionic thiolate form, which is well positioned for a nucleophilic attack on the allylic carbon 6 of the oxirane ring of LTA_4, forming a thioether bond. This opens the oxirane ring of LTA_4 simultaneously, and the resulting substrate oxyanion is stabilized by hydrogen bonding with the enzyme. Arg-104 might play a crucial role here as well. Eventually, the oxyanion is protonated, giving rise to LTC_4.

Microsomal Glutathione Transferase-2

MGST2 was identified in 1996 (201) as a close relative to FLAP and LTC4S (to which it displays 33% and 44% amino acid identity, respectively), and just like these two enzymes it is also involved in leukotriene biosynthesis. MGST2 is a 16.6 kDa membrane protein with a calculated isoelectric point of 10.4 (201).

Northern Blot analysis of the tissue distribution of MGST2 revealed that its mRNA is expressed in a wide variety of organs, like human liver, spleen, skeletal muscle, heart, adrenal gland, pancreas, prostate, testis, and fetal liver and spleen. In lung, brain, placenta, and bone marrow expression is very low.

MGST2 catalyses the conjugation of GSH to LTA_4 to yield LTC_4, however with lower affinity as compared to LTC4S (K_M values of 41 μM and 7 μM, respectively). MGST2 also catalyses the formation of a second product, displaying a conjugated triene UV absorption spectrum with a maximum at 283 nm, which elutes as a more polar compound than LTC_4 from the hydrophobic column material during separation by RT-HPLC. However, because the mass spectrum of this product is identical to the one of LTC_4, this second product is probably an isomer of LTC_4. This suggests that MGST2 has less catalytic stereospecificity compared to LTC4S. In addition, and in contrast to LTC4S, MGST2 is able to catalyse the conjugation of CNDB with GSH, though at a much lower rate compared to MGST1, and it furthermore possesses glutathione peroxidase activity, thus catalysing the reduction of 5-HPETE to 5-HETE (202).

Microsomal Glutathione Transferase-3

Although MGST3 is commonly regarded to form its own, evolutionarily distinct subgroup within the MAPEG superfamily, it is closely related to MGST2, with which it shares 36% sequence identity. MGST3 was discovered in 1997 (202) as a 16.5 kDa protein with a calculated isoelectric point of 10.2.

Similar to MGST2, MGST3 shows a wide tissue distribution with high expression of the mRNA in human heart, skeletal muscle, and the adrenal cortex. It was also found in

brain, placenta, liver, kidney, and in several glandular tissues such as pancreas, thyroid, testis, and ovary. In lung, thymus, and peripheral blood leukocytes, however, expression of MGST3 was very low.

MGST3 possesses glutathione-dependent peroxidase activity towards 5-HPETE, just like MGST2, and was also found to catalyse the production of LTC_4 from LTA_4 and GSH with low specificity. However, it is not able to use CNDB as a substrate for the glutathione transferase activity.

Microsomal Glutathione Transferase-1

MGST1 was identified long before the MAPEG superfamily was defined, and it is thus the first protein of this group of proteins described. Since its discovery, MGST1 was subject to a large number of studies, so that it can be regarded as a model for, and takes a leading role in the investigation of the whole superfamily, both with respect to enzymological and structural features.

Enzyme function in rat liver microsomes that conjugates GSH to 3,4-dichloronitrobenzene was described in 1961 (203) and later attributed to a distinct membrane bound glutathione transferase (204). In 1982 MGST1 was first purified and characterised (205-206). The rat protein consists of 154 amino acids, has a molecular weight of 17.3 kDa and an isoelectric point of 10.1 (207-209). It possesses both glutathione transferase and glutathione-dependent peroxidase activity and is involved in phase II reactions of the biotransformation process to protect the organism from toxic lipophilic substances and oxidative stress. MGST1 occurs in high amounts in hepatocytes, while significant levels have been detected in the intestine, the adrenal gland and testis. Low levels of expression were found in thymus, lung, spleen, kidney and brain (210). Its intracellular distribution in the liver concentrates on the endoplasmic reticulum and the outer membrane of mitochondria, where it accounts for up to 3% and 5%, respectively, of the total protein of isolated membrane fraction (210-211).

Besides restriction to GSH as an obligatory substrate, MGST1 displays wide substrate specificity with regards to the second, electrophilic substrate, and so far more than 25 such substrates have been characterised. However, LTA_4 serves only as a poor substrate for MGST1, unlike for the other MAPEG glutathione transferases MGST2 and MGST3 (202), and the product LTC_4 was identified as an inhibitor for MGST1. CDNB is usually used as an electrophilic substrate when MGST1 activity is tested in standard laboratory activity assays. Human (208, 212) and rat (207) MGST1 show specific activities towards CDNB of 1.9 and 2.0 $\mu mol\ min^{-1}\ mg^{-1}$, respectively.

A unique characteristic of MGST1, as compared to other MAPEG enzymes as well as other glutathione-S-transferases, is that MGST1 can be activated. However, activation has only been observed in the mammalian protein. Factors that can activate the enzyme range from radiation and heating to proteolytical cleavage of the N-terminal region, oxidative

and nitrosative stress, chemical modification by for instance sulfhydryl reagents, and ligand interaction (213). The conventional standard procedure to obtain activated MGST1 is treatment of the protein with NEM. A particular role in this context has been assigned for the only cysteine residue of MGST1 at position 49, which apparently acts as an antenna detecting cellular stress. This residue covalently binds to NEM (214).

The kinetic mechanism of MGST1 can be described by a slow GSH binding and thiolate formation step followed by a chemical step that depends on the reactivity of the electrophilic substrate. Since it is unlikely that the actual deprotonation of GSH is a slow process, it is believed that the proton-transfer itself is rapid but must be preceded by an obligatory, slow conformational transition and the formation of a tight enzyme-thiolate-complex (215).

Discussion

Methodology

In the following section a brief review of some of the methods that were used for the work in papers I to V will be presented. Only methods directly employed by the author of this thesis were selected to be discussed, and critical steps are highlighted. This section is considered to be a discussion of general aspects, as well as of advantages and disadvantages of the methods, rather than to provide practical information. Details about the specific methods can be found in the respective papers and the references therein.

Membrane Protein Over-expression

A prerequisite for any investigation of proteins is the availability of sufficient amounts of the protein of interest. This is of particular importance for structural studies, which in general require much more material than functional studies. Furthermore, the preparation of well-ordered two-dimensional or three-dimensional protein crystals, which is necessary for structural determination by electron crystallography or x-ray crystallography, respectively, requires pure and homogenous protein samples. Therefore, structural studies of proteins remain a challenge. This is true for structural elucidation of soluble proteins, but especially for those studies that focus on membrane proteins. The natural abundance of membrane proteins is in general too low to conveniently isolate sufficient amounts directly from cells or tissue. There are, however, notable exceptions of membrane proteins that naturally occur in large amounts in different cells or tissues, which can be obtained from animals or other sources. MGST1 is for instance one of these exceptions, as it may account for up to 5% of the total protein of isolated membrane fractions obtained from rat liver cells (210-211). Protocols for protein purification from these sources have been established (205-206), and sufficient amounts of MGST1 could be obtained from rat livers to elucidate the structure of this protein (181). Following this approach, however, one cannot obtain information about the human orthologues of the respective proteins.

If the protein of interest is not available from natural sources, it can be obtained by protein over-expression. Molecular biologists have developed different host organisms, plasmids and techniques for the molecular cloning of specific genes and the subsequent protein over-expression. This technology offers the possibility to express proteins from

one organism in a different host organism, known as heterologous protein over-expression, which is in many cases the best or only way to obtain adequate amounts of human protein.

Bacterial expression systems are very common, and one of the most successful host organisms used among the bacterial systems is the gram-negative rod-shaped bacterium *Escherichia coli*, which has been extensively studied.

Bacterial host organisms offer many advantages, including growth on inexpensive media, a large number of different host strains and expression vectors that match different requirements, rapid biomass accumulation, and simple process scale-up. Furthermore, they allow for rapid genetic manipulation (216-219). However, together with the archaea, bacteria belong to the prokaryotes, which generally lack subcellular compartmentalisation as it can be found in eukaryotes. Therefore, all membrane proteins to be obtained by over-expression must be inserted into the bacterial membrane. This might create a bottleneck during protein over-expression and can lead to the formation of inclusion bodies, i.e. insoluble protein aggregates that accumulate in the cytosol. Although over-expression into inclusion bodies, followed by subsequent protein refolding and purification, can be a good strategy to obtain the protein of interest (218), the risk is high that re-folding is not successful or that the re-folded protein lacks biological function. Furthermore, bacteria lack many of the enzymes that eukaryotic organisms use for post-translational modifications. The heterologously over-expressed protein can therefore differ from its natural form if post-translational modification occurs in the particular case.

Alternatives to the bacterial systems comprise eukaryotic expression systems such as yeast or insect cells, as well as cell-free expression systems.

The yeast host organism *Pichia pastoris* is of particular importance for the structural investigation of the MAPEG superfamily, because it was used for the expression of LTC4S, leading to one of the available MAPEG protein structures (183). This was the first time that heterologous protein over-expression was used to obtain a high-resolution structure of an integral membrane protein. Protein over-expression in insect cells such as the Sf9 cells from *Spodoptera frugiperda* was used for the expression of, among others, FLAP (191), MGST3 (202), and MPGES1 (220).

The advantage of cell-free expression systems is that both transcription and translation take place *in vitro* under tightly controlled conditions. This allows, for instance, the incorporation of specifically labelled amino acids, which is particularly useful for structural investigations employing NMR. However, cell-free protein expression is expensive and to date there are no reports in the literature regarding cell-free expression of members of the MAPEG family.

For the studies reported in papers I and II human and rat MPGES1 were heterologously over-expressed in *E. coli*. It was subsequently partially purified and used for inhibitor binding studies (paper II) or purified to apparent homogeniety to be used in electron crystallography (paper I). The protocols used for expression, subcellular fractionation and purification to homogeneity were previously established (34).

Site-directed Mutagenesis and Cassette Mutagenesis

Site-directed mutagenesis is a molecular biology technique that is used to introduce specific mutations at a defined site in a DNA molecule. These mutations are generated by polymerase chain reaction using oligonucleotide primers that are specifically designed to contain the desired mutation. The resulting DNA product shows the same mutation. Usually, the mutation affects only one codon that determines a specific amino acid in the resulting protein. Exchange of one, two or all three nucleotides in one codon results in a new codon and, when expressed, a different amino acid within the protein sequence. However, primers can be designed so that more than one codon will be affected and hence several amino acids are mutated at a time.

Cassette mutagenesis is a technique similar to site-directed mutagenesis; however, this approach generates a protein in which a large number of amino acids or a whole protein domain is exchanged. It involves the action of restriction endonucleases and DNA ligases for the exchange of a whole stretch of DNA in a plasmid and is therefore dependent on the presence of specific restriction sites.

Cassette mutagenesis and site-directed mutagenesis are versatile techniques that can be applied to diverse scientific questions. However, these questions always concern the functional role of one part of the investigated protein or the identification of a critical residue, e.g. in protein-protein interaction, in substrate or inhibitor binding, or in the catalytic mechanism of an enzymes.

The results of mutagenesis experiments might be hard to interpret, because it might not be trivial to plan good control experiments. Therefore, the design of a specific experiment is crucial. Site-directed mutagenesis is often used to exchange an existing residue, which fulfils a certain function, towards another residue, which has different characteristics and cannot fulfil this function. In this case, the resulting loss of function might be interpreted as an effect of the mutagenesis. However, it cannot be excluded that other factors out of the control of the experimenter influence the result, so that the effect of the mutation would be overrated. Such factors may include protein expression, membrane insertion or protein folding into the functional conformation. Better experiments with a more meaningful outcome will result in a gain or a change of function. It is likely that mutation toward residues with similar characteristics will yield this outcome; however, this is hard to predict and depends on the individual experiment.

In paper I single amino acid mutants of human MPGES1 were created by site-directed mutagenesis in order to validate the protein structure of MPGES1. Residues likely to have important structural functions or to be involved in cofactor binding were mutated in order to verify their predicted role. In contrast to this approach, paper II employs cassette mutagenesis to create chimeric forms of MPGES1 as well as site-directed mutagenesis in order to exchange amino acids that occur in human MPGES1 for those that occur at the same position in rat MPGES1. These mutagenesis experiments were conducted in order to investigate the difference between the enzymes of these two species with respect to inhibitor binding.

MPGES1 Activity Assays and Inhibitor Studies

MPGES1 catalyses the isomerisation of PGH_2 to PGE_2 (30). In order to measure this activity *in vitro*, an enzyme activity assay was developed (108). The principle of this assay is that different products, which result from a short incubation of PGH_2 with the enzyme, are separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and subsequently quantified by comparison of their UV absorption with an external standard curve.

In this classical PGE_2 synthase activity assay, the reaction takes place in single vials on wet ice. Due to the unstable nature of the substrate PGH_2 in aqueous solution, it is necessary to aliquot the acetone-dissolved substrate for each incubation prior to the assay and to keep the individual aliquots on dry ice until the assay starts. This helps to avoid non-enzymatic degradation of PGH_2 into PGE_2 and PGD_2 and thus to reduce the background. After the incubation is terminated by the addition of $FeCl_2$ solution, which decomposes the remaining substrate to 12(S)-hydroxy-5(Z),8(E),10(E)-heptadecatrienoic acid (12-HHT) and malondialdehyde (MDA) (221), solid-phase extraction (SPE) of the samples is necessary to prepare them for RP-HPLC separation. The extraction procedure makes it necessary to add an internal standard to each sample in order estimate the extraction efficacy. The samples are analysed in a sequential way by RP-HPLC separation and UV detection. In order to achieve baseline separation of the different analytes as well as the internal standard, every sample needs about 30 minutes to be analysed. Standards of different concentrations need to be processed in the same way as the samples. Finally, all chromatograms are integrated, and the area under the curve for each peak that is found in a chromatogram needs to be evaluated to obtain the result for a particular experiment.

This assay has strong advantages. It is versatile, and incubation conditions such as the reaction time and temperature can be easily varied. Enzyme from different sources can be used, and the requirements for enzyme purity and concentration are rather low. Furthermore, this assay allows for investigation of different substrates or co-factors, as well as for changes in their concentrations in order to determine characteristic enzyme parameters in steady state kinetic experiments. The products that are formed by the enzymatic reaction are directly identified by their characteristic retention time during liquid chromatography, and putative by-products are likely to be detected because they will appear as a distinct peak in the chromatogram. Finally, the assay is sufficiently sensitive to detect amounts in the low picomole range. If the instrumentation is available, the assay can be improved by coupling the RP-HPLC to a mass spectrometer. This enhances the specificity of the assay because the molecular weight or the specific mass transition, respectively, is exploited for analyte identification in addition to the retention time. Furthermore, mass spectrometers are usually about one to two orders of magnitude more sensitive than UV detectors, so that even amounts in the femtomole range can be detected and quantified.

On the other hand, there are some limitations of this assay. It requires expensive instrumentation such as an HPLC system as well as a UV detector or mass spectrometer, respectively, including technical know-how to operate these instruments. Furthermore, the incubation procedure in single vials, the sample work-up, and the analytic steps are

very labour intensive and time consuming, limiting the maximal number of samples that can be processed to about 48 per day.

Hence, despite its obvious advantages as a discovery tool, this classical PGE_2 synthase activity assay is not capable to meet the requirements of medium- to high-throughput applications that are preferentially used for screening tests during drug development or for other experiments that generate a large number of samples.

Therefore, a second, colorimetric activity assay was developed (222), which is performed in a 96-well plate format and thus capable to process more samples at a time. This second assay is based on the detection of a stable, fluorescent conjugate formed from 2-thiobarbituric acid (TBA) and MDA, a method that was previously described (223). Thus, whereas the classical PGE_2 synthase activity assay measures the increase of the product PGE_2 during the incubation time, the TBA assay measures the decrease of the substrate PGH_2 during the enzymatic reaction as reflected by the levels of MDA generated from the remaining substrate PGH_2 at the end of the enzymatic reaction.

In preparation of the TBA assay, appropriate enzyme dilutions, including buffer controls, boiled controls and positive controls, are aliquoted to the wells of a customary 96-well plate, which is kept on wet ice prior to the incubation. In order to start the incubation, the substrate PGH_2 is diluted with ice-cold buffer in sufficient amounts for all wells on the plate and added to the enzyme immediately after mixing. The incubation of substrate and enzyme can be performed optionally on wet ice or at room temperature. $FeCl_2$ solution is added to the wells after the desired incubation time and all unused substrate is converted to 12-HHT and MDA. In order to keep the non-enzymatic degradation of the substrate as low as possible it is important to start pipetting immediately after buffer and PGH_2 were mixed. Furthermore, one needs to rapidly proceed pipetting throughout the whole plate and aim to achieve a similar speed when adding the $FeCl_2$ solution after the incubation time. Use of an electronic multichannel micropipette is advisable to achieve reproducible results. Excess of TBA is subsequently added to the reaction mixture, the plate is sealed and incubated for 30 minutes at 80°C. MDA and TBA react under these conditions to form a pink coloured conjugate, which has an absorption maximum at 532 nm (224-225), and which is able to emit light at 545 nm when excited at 485 nm. A linear increase of absorption or fluorescence is observed with increasing concentrations of the MDA-TBA conjugate. Thus, in any given sample the relative concentration of this conjugate compared to the positive control and the boiled control, respectively, can be determined photo- or fluorometrically. The amount of the conjugate is proportional to the amount of MDA, which in turn equals the amount of unreacted PGH_2 that is left at the end of the incubation time. In other words, the amount of the conjugate is inversely proportional to the amount of PGE_2 formed.

The TBA assay has several obvious advantages compared with the classical PGE_2 synthase activity assay. It is a robust and technically less demanding assay that is generally applicable in most laboratories. Furthermore, a significantly increased number of samples can be processed, and the results are obtained immediately after readout of the plate.

The disadvantages of the TBA assay are however, that it does not provide absolute quantification of the enzymatic reaction, in contrast to the classical PGE_2 synthase activity assay. Also, because of its principle to measure the decrease of the substrate rather than the increase of the product, it is much harder to detect low enzymatic activity close to the background. With regards to the enzyme source as well as the enzyme concentration, the TBA assay makes higher demands than the classical assay, because high amounts of membrane fractions disturb both the photo- and fluorometric measurement due to turbidity from protein precipitation as well as adsorption of the MDA-TBA conjugate to these precipitates (224).

Inhibition of MPGES1 activity by specific small molecule inhibitors can be principally evaluated using both assays; however, the TBA assay is the method of choice because of its convenience. This assay is sufficiently sensitive to detect a robust decrease of enzymatic activity caused by small molecule inhibitors, and absolute quantification of the catalytic conversion is not necessarily needed. Usually, estimation of the fractional inhibition relative to the uninhibited protein is sufficient to provide the desired information. It should be noted that comparison of individual measurements is usually reliable as long as the measurements are carried out within the same experiments and on the same 96-well plate. Plate-to-plate comparison is possible, however, it should be avoided. Therefore, fractional inhibition is always expressed relative to an uninhibited control measured on the same plate. Within a certain concentration range the TBA assay is compatible with several chemicals and organic solvents that are frequently used during drug development, such as dimethylsulfoxide (DMSO). In order to obtain a full inhibition curve, several orders of magnitude in the concentration of the inhibitor must be covered. This results in a large number of individual measurements. A usual experiment consists of ten different inhibitor concentrations spanning from the picomolar range to the low micromolar range and is measured in four to eight replicates per concentration. The capability of the TBA assay to analyse many samples in a relatively short time makes it possible to handle all of these samples. Finally, certain parameters of the TBA assay such as substrate concentration and incubation time can be adjusted, allowing for investigation of the inhibition mode employed by different compounds.

The classical, RP-HPLC based activity assay was used in paper I to measure the activity of different MPGES1 mutants expressed in *E. coli*, as well as in paper III to assess the PGE_2 formation of microsomes obtained from different eukaryotic cancer cell lines. In paper II the TBA assay was used to investigate the inhibitor binding site of MPGES1 using two competitive enzyme inhibitors and several mutants as well as a rat/human chimeric form of MPGES1, which were expressed in *E. coli*.

Prostanoid Analysis

Prostanoids are of great importance in many aspects of physiology and pathophysiology. Defining the roles of the various prostanoids and other eicosanoids in human health and disease remains a challenging task in eicosanoids research. In order to answer these research questions or to monitor the effects of drugs on the *in vivo* synthesis of prostanoids in model animals as well as in man, it is critical to accurately determine their abundance in different biological samples. Two principally different methods are used for prostanoid quantification, immunoassays and separation techniques combined with different types of mass spectrometry (142).

Immunoassays employ antibodies specific for the prostanoid of interest. A limited number of binding sites is provided by the antibody, which is fixed in the wells of a 96-well pate for the assay. The analyte in the sample has to compete with a so-called tracer for these binding sites. The tracer is either a radioactively labelled analogue of the analyte, or the analyte itself, chemically coupled to an enzyme, which is capable forming a product with distinct absorption characteristics from a substrate that is added during the readout step. Depending on the type of tracer used, the assay is either called radio-immunoassay (RIA) or enzyme-immunoassay (EIA). The concentration of the competitive tracer is kept constant in both cases, so that the concentration of analyte in the sample will determine what fraction of the tracer will bind to the antibody. At low analyte concentrations a large fraction of tracer will bind and vice versa. After a certain incubation time all unbound molecules are washed away. The amount of bound tracer can now be measured, or traced, either by readout of the radioactivity on the plate or by addition of the substrate and subsequent photometric determination of the light absorption by the product. The analyte concentration is inversely proportional to the tracer and can be quantified with the help of a standard curve that is measured on the same 96-well plate. In order to avoid work with radioactive material, RIA has nowadays almost completely been replaced by EIA.

EIA are commercially available for many, but not for all prostanoids. These assays are usually reliable, sensitive and easy to use; however, every sample needs to be measured in several replicates and at an appropriate dilution, limiting the number of samples per plate.

On the other hand EIA provide no direct measurement of the analyte; the parameter that is actually measured is the absorption of a molecule formed by the tracer. This parameter might be prone to error because the readout is dependent on several assumptions and experimental steps. Furthermore, EIA are highly dependent on the specificity of the antibody used, and cross reactivity with other compounds is a common problem because of the large number and structural similarity of the different prostanoids.

Mass spectrometry has been a key technique in the discovery of eicosanoids, both in structural (13) as well as in quantitative (226-227) studies. Mass spectrometers are very powerful detectors because they are both sensitive and general. The principle of mass spectrometry is that ions in gas phase are separated according to their mass to charge ratio (m/z) and subsequently detected. Generally, all ionisable compounds can be analysed by mass spectrometry; the ionisation step is, however, critical. This is true especially for the

analysis of complex biological samples, because in these samples analytes present in liquid phase must be transformed to yield ions in gas phase.

Gas chromatography combined with detection by mass spectrometry (GC-MS) was initially used for quantitative analysis of eicosanoids with high specificity and sensitivity (228-229). In GC-MS the ionisation step is less critical, because the analytes are already in gas phase. However, in order to make analytes from aqueous biological samples volatile, different derivatisation procedures during the sample preparation are usually required. GC-MS is therefore very time-consuming, and due to thermal instability of many of the eicosanoids it is not an ideal analytical technique.

During the last few decades several soft atmospheric pressure ionisation techniques have been developed, such as atmospheric pressure chemical ionisation (APCI) (230), atmospheric pressure photoionisation (APPI) (231), and electrospray ionisation (ESI) (232). These techniques make it possible to couple liquid chromatography with mass spectrometers because they are capable of producing gas phase ions from analytes in aqueous solution (233). In addition, these ionisation techniques are considered 'soft', because they produce little-to-no in-source fragmentation of the analyte ion, which makes them especially suitable for the investigation of biomolecules. Nowadays, LC-MS or LC-MS/MS is used for the analysis of eicosanoids in the vast majority of cases, and the most commonly used ionisation technique during these LC-MS analyses is ESI, which was proposed in 1968 (234) but not applied in biochemical analyses until 1984 (235).

In LC-ESI-MS, ionisable molecules in liquid phase are brought into the gas phase by pumping the sample solution through a narrow capillary. A high voltage is applied between the capillary and the sampling orifice of the mass spectrometer. The polarity of this voltage can be set to either positive or negative mode, depending on the nature of the ionisable groups of the analyte. Because all eicosanoids are derived from fatty acids and thus contain a carboxyl group, they are usually analysed in negative mode. At the negatively charged capillary wall all positively charged counter ions become discharged, while the negatively charged analyte ions remain in solution. Heated nitrogen gas is used to assist the formation of a spray of small, highly charged droplets. The solvent of these droplets evaporates under the influence of the gas and the high temperature until coulombic repulsion forces between the analyte ions of identical charge overcome the surface tension and the droplets become unstable and break to give rise to smaller droplets. Two theories have been postulated for the final generation of desolvated ions. According to the first theory, the ion evaporation model (236-237), sufficiently small and volatile ions can evaporate from the surface, giving rise to gas phase ions. The second theory, the charged residue model (234), postulates that the process of solvent evaporation, coulombic repulsion, and droplet fission continues until droplets are reached that contain only a single ion. Further evaporation eventually gives rise to gas phase ions.

After gas phase ions are formed they are introduced into a mass analyser, where they are separated according to their m/z ratio. Several types of mass analysers are available, including quadrupole (Q) and triple-quadrupole (QQQ) instruments, time-of-flight (TOF) analysers, ion trap instruments and fourier transform ion cyclotron resonance (FTICR) mass spectrometers. These different instruments have specific characteristics, such as

mass accuracy, mass range, resolution, sensitivity, speed, and running expenses, and the choice of analyser is largely dependent on the specific application. For the quantitative analysis of eicosanoids Q and QQQ instruments are particularly suited and most commonly used because they combine a sufficiently high mass accuracy with a high sensitivity.

A quadrupole analyser is constructed of four electronically conducting cylindrical rods, which are arranged parallel to each other as two opposing pairs. They are cyclically charged with positive or negative direct current (DC), and variable radio frequency alternating current (AC) potentials are applied to each pair of rods. Gas phase ions are accelerated into the channel between the rods, and the AC potentials can be set so that only ions of a desired m/z ratio have a stable trajectory through the electric field of the quadrupole system. Thus, a quadrupole analyser can be used as a mass filter that allows only ions of interest to pass while all other ions are filtered out. In a QQQ instrument three of these single quadrupole systems are arranged in a linear way. The first quadrupole system is used to select ions of a m/z ratio corresponding to the analyte in the biological sample, the so-called mother-ion. These ions are introduced into a second quadrupole system, the collision cell. Here, the potentials are set so that all ions can pass; however, they encounter molecules of an inert gas such as argon in the collision cell. The kinetic energy of the analyte ions causes them to break upon impact on the argon molecules, a process known as collision-induced dissociation (CID). The CID process gives rise to specific molecular fragments, or daughter-ions, which are transferred to the third quadrupole system, where the AC potentials are set to select for the m/z values specific for these daughter-ions, which can pass and subsequently be detected. A pair consisting of mother-ion and daughter-ion is called the mass transition, which is very specific for a given molecule and can usually be detected with a low background noise, which characterises the excellent specificity and sensitivity of QQQ instruments. Several of these transitions can be measured after each other within millisecond time frames. This mode of action of a QQQ instrument is called multi reaction monitoring (MRM), which is most commonly used for quantitative analysis of eicosanoids.

Albeit QQQ instruments are highly specific, especially when operated in MRM mode, analysis of eicosanoids is still challenging because of the existence of many stereoisomeric molecules with identical mass that give rise to similar mother-ions and, in some cases, even to similar daughter-ions. Therefore, thorough separation of these compounds by liquid chromatography is required before mass spectrometric analysis. Furthermore, due to the fast metabolism of eicosanoids *in vivo* the choice of the analyte is critical and depends on the specific application.

LC-MS/MS methodology was used for quantitative analysis of primary prostanoids in cardiac tissue in paper IV and for determination of systemic PGE_2 production, quantified by the levels of urinary tetranor-PGEM in paper V.

Results

This thesis comprises five different studies regarding the structure and function of MPGES1. The results of most of these studies have already been published, and the publications can be found at the end of the thesis as papers I to IV. The last study, included as paper V, has not yet been published and is available in form of a manuscript.

Paper I and paper II deal with structural aspects of MPGES1, while papers III, IV, and V rather cover functional aspects of this enzyme in connection with different disease states. Here, the main findings are presented and discussed in the context of the current literature.

MPGES1 Structure

Elucidation of the protein structure of MPGES1 at 3.5 Å by electron crystallography is reported in paper I.

Human MPGES1 with an N-terminal poly-histidine tag was heterologously expressed in *E. coli* BL21(DE) pLysS. It was subsequently solubilised from the membranes using the non-ionic detergent Triton X-100 and purified to apparent homogeneity using both hydroxyapatite affinity and immobilised metal ion affinity chromatography. The detergent was slowly replaced with bovine liver lecithin during two-dimensional crystallisation dialysis, yielding highly ordered crystals of catalytically active MPGES1. Electron diffraction patterns were recorded from these crystals at various angles and used to calculate the three-dimensional enzyme structure at 3.5 Å. In order to validate the calculated protein structure, single amino acid mutations were introduced at positions within the protein anticipated to be important for the structural integrity or the binding of the essential cofactor GSH. Catalytic activity of these mutants was determined and compared to the activity of the native enzyme.

The subunits of MPGES1 are organised as a homotrimer. Each subunit consists of a bundle of four membrane spanning α-helices. The highly conserved TM2s from each subunit come together to form an inner core structure, which is surrounded by the remaining transmembrane helices. Intersubunit contacts are made by residues from TM2, TM1, and TM4. The binding site of GSH was identified between two adjacent subunits in a pocket deep within the homotrimer, so that it is inaccessible from the membrane as seen in the determined structure. Three molecules of GSH per trimer are found to adopt a U-shaped conformation. Residues from all four transmembrane helices are involved in GSH binding. Arg-70 from one and Arg-38 from an adjacent subunit make salt bridges to the carboxyl groups at either side of GSH. Arg-126, Tyr-130, and Tyr-28 were found in close proximity to the cysteine moiety of GSH. Other residues involved in GSH binding include Tyr-117, His-113, Arg-110, Glu-77, and His-72. Site-directed mutagenesis of some of these residues resulted in reduced or abolished enzyme activity.

The structures of MPGES1 and MGST1 (181) are closely related to each other. This is due to the high degree of sequence homology between the two proteins.

The overall structure of MPGES1 is also in agreement with the structures of FLAP (190) and LTC4S (182-183), indicating a common fold for all members of the MAPEG protein superfamily. However, the cytosolic loop between TM1 and TM2 is shorter in these two proteins as compared to MPGES1 and MGST1. Furthermore, FLAP and LTC4S display both an extra α-helix in addition to the four transmembrane helices, which is located at the N-terminus for FLAP (190), and at the C-terminus for LTC4S (182-183). The role of these extra helices is unclear. It is assumed that they are situated on the luminal side of the membrane of the endoplasmic reticulum, and it is therefore unlikely that they are involved in the interaction of FLAP or LTC4S with 5-LO, which is located in the cytosol.

Both electron crystallography (181, 184) and x-ray crystallography (182-183, 190, 238) have been used for the structural elucidation of members of the MAPEG protein superfamily. In general, x-ray crystallography is considered to yield protein structures at higher resolution, whereas electron crystallography is regarded as a technique to fairly rapidly assess the quaternary structure of proteins in two-dimensional crystals at low resolution, but it requires significantly more time and effort to obtain high resolution structures of membrane proteins using this technique. However, during recent years the resolution of membrane protein structures obtained by electron crystallography has improved. The advantage of this technique in contrast to x-ray crystallography is that membrane proteins in a two-dimensional crystal are located within a phospholipid bilayer, which resembles the natural environment of membrane proteins much closer than detergent micelles. In agreement with this, the structure of MPGES1 displays essentially no charged residues that are exposed to the hydrophobic core of the phospholipid bilayer, whereas more such charges are visible in the x-ray structure of LTC4S. Thus, these two techniques seem to give complementary results. X-ray structures might provide more details by being able to reach almost atomic resolution, while electron crystallography structures might show protein conformations that better match the *in vivo* situation. Both of these techniques are limited in that the models they yield are rigid snapshots of the protein that do not provide any information about the dynamic conformational changes. Structural data obtained by nuclear magnetic resonance (NMR) techniques could provide such information and complement existing knowledge.

Inhibitor Binding Site of MPGES1

In paper II the inhibitor binding site was investigated using a selective MPGES1 inhibitor that is active in the human enzyme but does not show an effect on the rat orthologue. This different binding characteristic became apparent during the drug development process, when the newly developed inhibitors were tested in different cell culture systems or animal models, respectively. The same characteristic is shown by other selective inhibitors of MPGES1, such as MF-63, which potently inhibits human MPGES1 with an

IC_{50} value of 1.3 nM but fails to inhibit the rat or the mouse enzymes (176). Interestingly, MF-63 proved to be active in guinea pig, where it displayed an IC_{50} value of 0.9 nM, a similar inhibition as in the human enzyme. Comparable species effects have also been observed for FLAP inhibitors (185).

The compound used in this study is a competitive inhibitor of MPGES1, i.e. it binds to the same site in the enzyme as the substrate PGH_2, and inhibition can be overcome by increase of the substrate concentration.

In order to investigate the species difference in inhibitor binding we created rat/human chimeric forms of MPGES1 by exchanging residues 115-140 from the rat enzyme to the human enzyme and vice versa, giving rise to the two chimeric proteins, hum115rat140hum and rat115hum140rat, respectively, which displayed similar specific activities as the rat wt enzyme. The exchanged residues comprise the end of TM3, the second cytosolic loop between TM3 and TM4, as well as the initial half of TM4. Thus, the crucial interface between two subunits in the trimer, i.e. the proposed active site of MPGES1, was targeted with this approach. When tested for inhibition, the compound failed to interfere with the catalytic activity of the chimeric enzyme hum115rat140hum but gained inhibitory potential in the chimeric enzyme rat115hum140rat, indicating that some of the residues that differ between the human and the rat enzyme within this part of the protein play a crucial role for inhibitor binding. These residues were subsequently targeted by site-directed mutagenesis in order to investigate whether the resulting single amino acid mutants within the rat enzyme were inhibited by the compound. None of the mutations alone conferred inhibitor sensitivity to the rat enzyme, however, a combination of three mutations in the rat enzyme, V131T, F135L, and F138A, showed inhibition by the compound, albeit with a higher IC_{50} value compared with the human wt or the chimeric enzyme. When the same residues were mutated in human MPGES1 towards the residues in rat, the resulting mutant was insensitive to the inhibitor. Amino acid changes within TM1 opposite to the position of the three residues apparently do not play a crucial role in inhibitor binding, as mutation of these residues did not change the IC_{50} value of the chimeric enzyme.

In the structure model of MPGES1 the three identified residues align at the same side of TM4, all being one turn of the helix apart from each other. They are thus lining the entrance of the crevice between TM1 and TM4 of adjacent subunits, which is believed to be the entrance to the putative active site of MPGES1 (Figure 10A). Whereas the three residues are all relatively small in the human enzyme, allowing the inhibitor to enter the active site, they were substituted for bulky aromatic residues in two out of three positions in the rat enzyme. Apparently, these substitutions sterically hinder the inhibitor from access to the active site, while the substrate PGH_2 is more flexible and thus able to enter. It seems that dynamic opening of the structure to give access to the active site in MPGES1, as suggested in paper I, is not sufficient in the rat enzyme to let the inhibitor pass these residues with gatekeeper function. The same substitutions in TM4 were found in the mouse orthologe, but not in guinea pig, which can explain why the inhibitor MF-63 is active in this species (176). Furthermore, the three residues with gatekeeper function in MPGES1 correspond to the residues that were proposed for substrate binding in LTC4S

(182-183) as well as to the residues that are involved in inhibitor binding of FLAP (190). To date, FLAP is the only member of the MAPEG protein superfamily on which structural information of an inhibitor binding site is available.

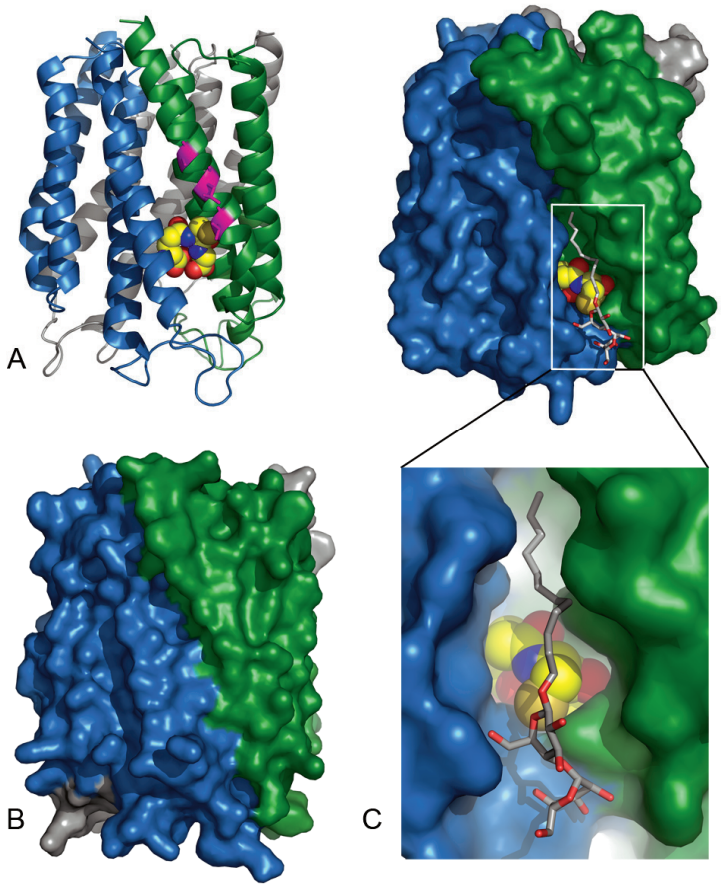


Figure 10. Indications for the location of the active site of MPGES1. *A. Residues Thr-131, Leu-135, and Ala-138, depicted in stick model representation and coloured in magenta, are aligned in TM4 at the entrance to the cleft between two subunits. In the rat enzyme, these residues are more bulky than in human MPGES1 and occlude the entrance to the active site. Some competitive MPGES1 inhibitors can therefore not bind to the rat enzyme. B. In the experimentally obtained structure of MPGES1, TM1 and TM4 of two adjacent subunits are close together, and GSH is not accessible from the membrane. C. On the contrary, in a homology model of MPGES1, which is based on the LTC4S structure, the two helices reveal an opening from the lipid bilayer, which provides a natural entry point to the active site. PGH_2 is modelled into the resulting cleft, depicted in stick representation with carbon atoms in grey. It is likely that MPGES1 alternates between an open and a closed conformation during the catalytic mechanism in order to give access to its active site. The proteins are depicted with their luminal side up.*

Active Site and Catalytic Mechanism of MPGES1

Several lines of evidence suggest that the active site or the inhibitor binding site, respectively, of all MAPEG members is highly conserved. This includes the location of GSH in MGST1 (181), LTC4S (182-183), and MPGES1 (paper I) between two adjacent subunits of a trimer, the location of the inhibitor MK-591 that was found at a corresponding position in the structure of FLAP (190), the presence of a detergent molecule that was found to bind in the crevice between two adjacent subunits in the structure of LTC4S and that was postulated to mimic the substrate LTA_4 (183), as well as the presence of gatekeeper residues that regulate access for competitive inhibitors to the active site in rat MPGES1 (paper II).

When the structure models of MPGES1 and LTC4S were compared, it became apparent that the structure of LTC4S is much wider and reveals an opening in form of a V-shaped cleft between TM1 of one subunit and TM4 of an adjacent subunit, which allows for access to GSH from the cytosolic leaflet of the membrane (182), (183) (Figure 8). No such entry point was found in the structure of MPGES1, where TM1 and TM2 are closer together (Figure 10B). However, it seems plausible that the substrate PGH_2 enters the active site, indicated by GSH, via the membrane. PGH_2 is formed by COX, which is located at the luminal side of the endoplasmic reticulum. Furthermore, PGH_2 is a hydrophobic molecule that is assumed to readily diffuse through the membrane, where its labile endoperoxide moiety would be protected from hydrolytic degradation. As no alternative entry point for PGH_2 is evident, the determined structure is interpreted as a closed conformation of the enzyme, and a dynamic opening of the protein is suggested to be part of the catalytic mechanism in order to allow PGH_2 to access the active site. A model for an open form of MPGES1 was generated by homology modelling of MPGES1 with the structure of LTC4S as a template. In the open conformation, GSH is accessible from the membrane (Figure 10C), and it is apparent that the cytoplasmic part of TM1 occludes the active site in the experimentally determined closed conformation. Open and closed conformation of MPGES1 can be converted into each other by displacement of the cytoplasmic part of TM1 around a hinge region, which is indicated by the ion bond between Lys-26 and Asp-75. Mutation of Asp-75 results in loss of enzyme function (unpublished results). Strictly limited access to the active site of MPGES1 might also be an explanation for its limited substrate specificity in contrast to, for instance, MGST1, and for the fact that the co-factor GSH is not consumed during the catalytic cycle (34).

In conclusion, these findings argue for a highly conserved active site and/or inhibitor binding site throughout the MAPEG protein superfamily. The location of the binding site facilitates entry via the phospholipid bilayer of the membrane of the various hydrophobic substrates and competitive inhibitors, which are mostly also hydrophobic.

In 1967, Hamberg and Samuelsson found that incubation of whole homogenates of sheep vesicular gland with DGLA, which was tritium labelled at carbon 9, resulted in loss of most of the tritium label during the formation of PGE_1, whereas DGLA with tritium

label at carbon 11 gave rise to PGE_1 that retained most of the tritium label (79). Thus, the transformation of PGH_1 to PGE_1 must involve abstraction of the hydrogen at carbon 9.

A mechanism for the formation of PGE_2 was proposed, in which a GSH-assisted shift of a hydride to an adjacent incipient oxonium atom at carbon 9 occurs (239). However, because the resulting intermediate in this reaction would be a metastable thiohemiketal rather than a stable thioester it is not very likely that the reaction proceeds according to this mechanism.

It is more likely that the formation of PGE_2 proceeds either via a concerted acid-base-mechanism or a GSH peroxidase-like mechanism. Both mechanisms involve activation of reduced GSH, which is in line with the observation of a Meisenheimer complex with MPGES1 (unpublished results).

According to the concerted acid-base-mechanism (Figure 11A) isomerisation of PGH_2 to PGE_2 would occur by proton abstraction at carbon 9, facilitated by a base such as the thiolate anion of GSH, while at the same time an acid donates a proton to the peroxide oxygen at carbon 11.

Because MPGES1 also displays GSH-dependent peroxidase activity toward other hydroperoxides (34) the GSH peroxidase-like mechanism (Figure 11B) is favoured over the concerted acid-base-mechanism. The scenario of the GSH peroxidase-like mechanism involves the attack of the GSH thiolate at the endoperoxide oxygen at carbon 9. This leads to a GSH thioester as the transition state in the reaction. A proton donor is required to generate the hydroxyl group at carbon 11, and a proton acceptor to abstract the proton at carbon 9. Consequently, the GSH thioester bond would be broken in favour for the formation of a keto group at carbon 9 and GSH in its thiolate state would be thus recovered. In the structural model of MPGES1 only Arg-126, Tyr-28, or Tyr-130 are in reasonable distance to the thiol group of GSH for being able to participate in the reaction mechanism.

A similar mechanism was proposed for the formation of PGE_2 by MPGES2. Based on the crystal structure of MPGES2 and data obtained from mutagenesis studies (240) a model of the enzyme bound to its substrate was built, and a possible catalytic mechanism was deduced (105). PGH_2 seems to bind to a bowl-shaped cavity of MPGES2, in which two hydrophobic pockets can take up the α- and ω-chain. Residue Cys-110, which was shown in mutagenesis experiments to be essential for catalytic activity, is placed at the bottom of the cavity. Binding of PGH_2 orientates its endoperoxide moiety towards the side chain Cys-110. Together with Tyr-107, Cys-113, and Phe-112, Cys-110 is involved in a hydrogen bond chain, which lowers the pK_a of its sulfhydryl group. The model suggests that the proton of this sulfhydryl group is transferred to the peroxide oxygen at carbon 11of PGH_2, and the resulting thiolate anion of Cys-110 subsequently attacks the peroxide oxygen at carbon 9, forming a C-9 sulfenate ester. A water molecule, situated near the side chain of Tyr-107, is polarised by the hydroxyl group of Tyr-107 and thus able to abstract the hydrogen at carbon 9 from PGH_2. At the same time the ester bond between Cys-110 and the oxygen at carbon 9 of PGH_2 is broken and the keto group of PGE_2 is formed.

In LTC4S residue Arg-104 was proposed to both activate GSH to its anionic thiolate form and to act as catalytic residue (183). The corresponding residue in MPGES1 is Arg-126. We suggested in paper I that this residue might stabilise the GSH thiolate and act both as a proton donor and acceptor during the catalytic mechanism. When Arg-126 was mutated the PGE_2 synthase activity was lost or strongly reduced, but instead the resulting mutants were able to form $PGF_{2\alpha}$ (241).

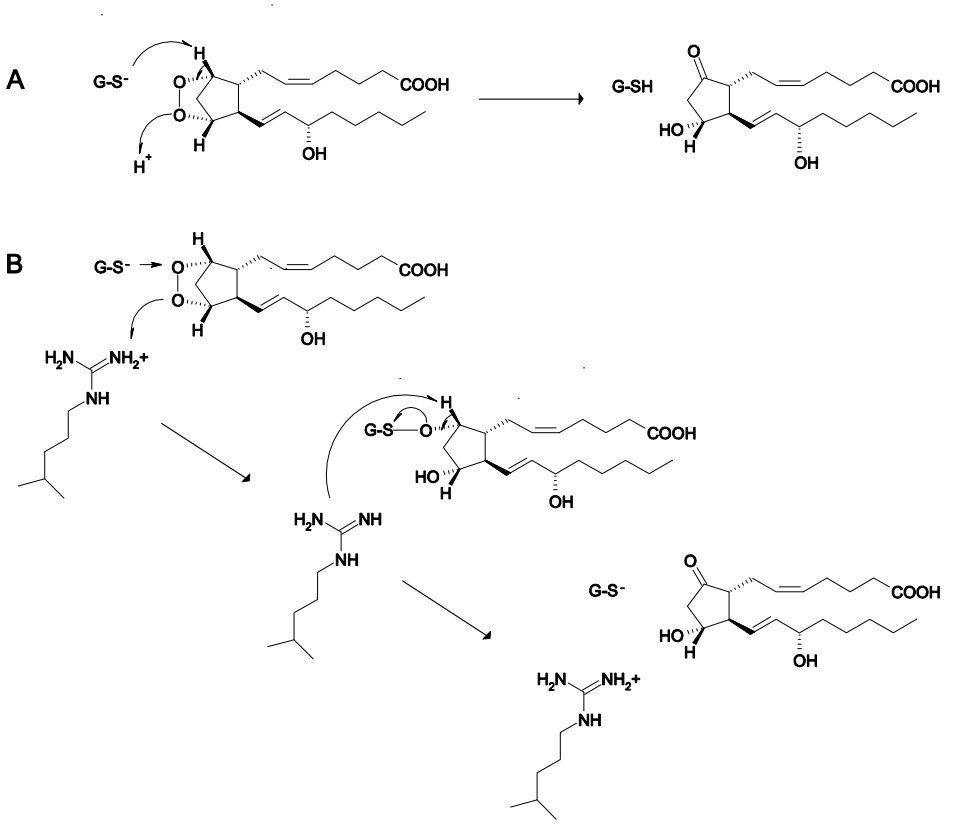


Figure 11. Proposed catalytic mechanism for MPGES1. *Two catalytic mechanisms for the formation of MPGES1 have been suggested. A. concerted acid-base-mechanism. B. glutathione peroxidise-like mechanism. G-SH and G-S- indicates glutathione in its thiol and thiolate form, respectively. In B. an arginine residue, such as Arg-126, is depicted, which might both donate a proton to oxygen 11 and accept a proton from carbon 9.*

Role of MPGES1 in Tumour Growth

Because eicosanoids in general and PGE_2 in particular have been shown to be involved in growth and survival of several types of cancer, the role of MPGES1 for the promotion of human prostate and lung cancer cells has been investigated in paper III.

High constitutive expression of MPGES1 was found in the human prostate cancer cell line DU145. In the human lung adenocarcinoma cell line A549, MPGES1 is inducible by IL-1β (30). Furthermore, both cell lines also displayed constitutive expression of COX-2, which are strongly induced in A549 cells and weakly induced in DU145 cells when stimulated with IL-1β. Stable transfection of the two cell lines with shRNA plasmids resulted in silencing of MPGES1 expression and significantly decreased PGE_2 synthase activity of microsomes isolated from the knock-down clones. When these cells were subcutaneously injected into the hind flanks of nude mice, only about half of the mice developed tumours from DU145 knock-down cells, and in only one fourth of the cases tumours developed from A549 knock-down cells. Furthermore, the time to develop a tumour size of about 0.2 ml was significantly prolonged in experiments with both DU145 and A549 knock-down cells compared to their respective wt controls. *In vitro* experiments showed a significantly reduced clonogenic capacity of the knock-down clones from both cell lines compared to their wt controls as well as significantly increased apoptosis in response to genotoxic stress for DU145, but not for A549 knock-down cells. In human prostate tissue obtained from surgery, MPGES1 expression seemed to be more abundant in tumour tissue than in prostate tissue with benign hyperplasia. Strong expression of MPGES1 was found in two out of five examined samples, and weak expression could be detected in the remaining three samples. In contrast to this, only one out of five benign control samples expressed low levels of MPGES1. COX-2, on the other hand, did not appear to be preferentially expressed in any of these two groups, as it was found in three of the malignant and in four of the benign tissue samples.

High expression of MPGES1, resulting in increased amounts of PGE_2, has been observed in colorectal cancer, lung cancer and endometrial carcinoma (242-244), and epidemiological evidence indicates that chronic use of NSAIDs lowers the incidence and mortality for colorectal cancer in humans and in animal models (245-247). Therefore, NSAIDs can be used in addition to conventional therapeutic approaches like surgery, chemotherapy and radiation therapy to treat cancer. However, the use of NSAIDs might be associated with severe adverse drug effects (160, 169), and the outcome of such additional therapy depends on the susceptibility of the cancer cells (248-249). Expression levels of MPGES1 in different prostate cancer cell lines varied considerably from high protein amounts in DU145 cells to lower levels in PC3 cells. In LNCaP cells MPGES1 expression was not detectable in line with low capacity to form PGE_2 *in vitro*. Similarly, different levels of MPGES1 expression have been demonstrated in the clinical samples of human prostate cancer tissue examined in this study, reflecting different degrees of susceptibility toward treatment with NSAIDs or selective MPGES1 inhibitors.

While COX-2 expression was found to be more equally distributed between prostate tumour tissue and prostate tissue with benign hyperplasia, MPGES1 expression appeared to be more abundant in malignant than in benign tissue. This indicates that MPGES1 expression in tumours might be associated with a higher degree of cell transformation during the process of carcinogenesis.

Taken together, results from *in vitro* and *in vivo* experiments demonstrate that targeted knock-down of MPGES1 in DU145 and A549 cells resulted in slower-growing cells, reflecting a less malignant phenotype. This may indicate that additional treatment with NSAIDs might prove to be beneficial in some cases of prostate cancer and furthermore, that MPGES1 may constitute an alternative therapeutic target in the treatment of prostate cancer.

Role of MPGES1 in Tissue Remodelling after Myocardial Infarction

Cardiovascular diseases are a leading cause of death (250). At the same time, NSAIDs are among the most commonly used pharmaceuticals worldwide, and selective inhibition of COX-2 is associated with an increased risk of cardiovascular events. It is therefore expectable that many patients at risk to develop cardiovascular complications might take MPGES1 inhibitors, once they are available on the market. In order to learn more about the role of MPGES1 in myocardial infarction (MI), the consequences that MPGES1 inhibition might have after MI were investigated in paper IV.

Acute coronary artery thrombosis was simulated in MPGES1 knock-out mice and their wt controls by ligation of the left anterior descending coronary artery, and the cardiac function as well as the heart tissue were investigated at several time points after the ligation. While the survival rate and the size of the infarcted tissue did not differ between the two groups, the indexes of left ventricular systolic and diastolic function were significantly worse in knock-out mice compared to their wt controls 28 days after the MI. The volume of the left ventricle was greater in knock-out mice than in the wt controls. Both knock-out and wt mice responded to the MI by cardiomyocyte hypertrophy, but in contratst to wt controls, the length-to-width ration of the cardiomyocytes from knock-out mice was greater, indicating that these cells underwent eccentric hypertrophy after MI. Furthermore, mRNA for several molecular markers of cardiomyocyte hypertrophy was more elevated in the knock-out mice, and multiple signalling pathways that regulate cardiomyocyte hypertrophy were modulated on the protein level after MI in these animals. The levels of both COX-2 and MPGES1 were increased on the mRNA level in the cardiac tissue after MI, and expression of these two proteins involved in PGE_2 formation could be located to inflammatory cells, but not to cardiomyocytes using immunohistochemical analysis. Before MI, the levels of all five primary prostanoids in the left ventricle and in the infarct zone were similar in wt and knock-out mice. After MI, the levels of these lipid mediators were found to be significantly increased in both groups of animals and tissue samples. However, in the infarct zone the prostanoid levels were roughly 10 times higher than in the left ventricle remote from the infarct. While no

significant difference between the wt and knock-out mice could be detected for PGD_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, and TXB_2, the levels of PGE_2 were significantly lower in the knock-out mice at 3 and 7 days after MI. At 28 days after MI, this difference in PGE_2 between the wt and knock-out mice was no longer statistically significant; however, the levels of PGE_2 were still elevated in both groups compared to the levels before MI.

Hypertrophy is the growth of an organ due to enlargement of its constituent cells rather than due to increase of the cell number, while the size of the cells remains approximately the same, which is known as hyperplasia. In the heart, hypertrophy is an adaptive response to several cardiovascular diseases in order to sustain cardiac function and to prevent heart failure (251). Concentric hypertrophy in response to a pressure load leads to cardiomyocyte thickening, while eccentric hypertrophy in response to a volume load leads to cardiomyocyte lengthening. After MI, stretched and dilated infarcted tissue increases the left-ventricular volume with a combined volume and pressure load on non-infarcted areas. All three types of adaptive response are referred to as remodelling, i.e. as the changes in ventricular topography, occurring both acutely and chronically after infarction (251-252). PGE_2 was shown to be important for cardiomyocyte hypertrophy *in vitro* (253-254), and cardiac ischemia-reperfusion injury in mice deficient in the PGE_2 receptor EP_4 resulted in an increase of infarct size and greater functional and biochemical derangements compared to wt controls (255).

The results from paper IV show that deletion of the gene coding for MPGES1 did not affect the infarct size; however, cardiac function was impaired 28 days after MI, and eccentric hypertrophy of viable cardiomyocytes remote from the infarction zone was more pronounced during tissue remodelling in the knock-out animals. COX-2 and MPGES1 were expressed in inflammatory cells in the infarct zone of wt animals, resulting in significantly higher levels of PGE_2 after MI. Therefore, it is likely that inflammatory cells infiltrate the infarct zone after MI and produce PGE_2, which might diffuse to the non-infarcted tissue and regulate hypertrophy. In MPGES1 deficient mice, lack of PGE_2 might lead to eccentric hypertrophy. Because the PGE_2 levels were about fourfold higher in the wt mice, the majority of PGE_2 formation is probably attributed to MPGES1 rather than to MPGES2 or cPGES, which are also expressed in the cardiac tissue.

Interestingly, cPGES mRNA levels were found to be transiently up-regulated after MI. Except for brain tissue, where LPS leads to an increase in PGE_2 synthase activity due to cPGES, this protein is generally believed to be constitutively expressed (98).

The levels of 6-keto-$PGF_{1\alpha}$ and TXB_2, reflecting the production of PGI_2 and TXA_2, respectively, were similar in the cardiac tissue of mice deficient in MPGES1 and in wt mice after MI. Studies using mice deficient in both MPGES1 and the receptor for low density lipoprotein (LDLR) show in contrast that urinary tetranor-PGEM was depressed, whereas urinary 2,3-dinor-6-keto $PGF_{1\alpha}$, but not 2,3-dinor-TXB_2 was increased (256-257). However, these results do not necessarily contradict each other, because the metabolites measured in paper IV reflect local prostanoid levels in the heart, whereas the urinary prostanoid metabolites reflect systemic biosynthesis. Systemic increase in 2,3-dinor-6-keto $PGF_{1\alpha}$ might be associated with protective effects on the cardiovascular

system. In the study presented in paper IV levels of urinary prostanoids were not investigated.

In conclusion, the data from paper IV suggest that MPGES1 plays a pivotal role in PGE_2 formation after MI, which seems to regulate cardiac tissue remodelling. Deletion of MPGES1 resulted in eccentric hypertrophy of cardiomyocytes, dilation of the left ventricle, and impaired cardiac function after MI. Therefore, use of MPGES1 inhibitors by patients at risk to develop MI should be considered with caution.

Consequences of MPGES1 Inhibition

Current medical science aims to improve treatment for patients by using knowledge obtained by molecular biology and other techniques to identify molecular targets whose biological activity is pathogenic or associated with disease. Target-based drug discovery subsequently screens for compounds to interfere with the activity of the molecular target and develops these compounds further for tests *in vitro*, *in vivo*, and eventually in clinical trials and practice. Such rational drug development has led for instance to a new class of anticancer medicine, the tyrosine kinase inhibitors (258).

With the discovery of MPGES1, a novel target for the development of anti-inflammatory drugs has been identified (30). The process of drug development to gain MPGES1 inhibitors is currently ongoing. As an enzyme, MPGES1 constitutes an attractive target, because small molecule drugs that interfere with enzyme activity are usually also available for oral administration, the most desirable course of treatment. In fact, nearly half of all marketed small molecule drugs inhibit enzymes as their molecular target (259).

Experience with NSAIDs and especially with COXIBs has raised awareness for possible adverse drug effects associated with the interference in the interconnected network of lipid mediators. COXIBs were developed to specifically inhibit COX-2 as a molecular target, similar to the current development of inhibitors directed against MPGES1. COXIBs were thought to be more specific and connected with fewer side effects than the available traditional NSAIDs. However, molecular mechanisms that were discovered with the emergence of COXIBs limited their use.

Evidence from *in vivo* studies using knock-out mice suggests a more positive outcome of MPGES1 inhibition. MPGES1 knock-out animals, unlike COX-2 knock-out animals, have a normal phenotype (37, 260-262).

Basal physiological levels of PGE_2 produced by constitutively expressed PGE_2 synthases are not expected to be affected by MPGES1 inhibitors, which interfere only with induced PGE_2 formation under pro-inflammatory conditions. In the study in paper IV basal levels of PGE_2 were indeed detected within tissue of MPGES1 knock-out mice.

Whereas inhibition of COX-2 prevents the production of PGH_2, which is the substrate for several terminal prostanoid synthases, inhibition of MPGES1 does not affect levels of PGH_2. Different hypotheses predict that shunting effects will occur when MPGES1 is

inhibited in order to redirect PGH_2, which is formed in high amounts when COX-2 is up-regulated under inflammatory conditions. Evidence from work with fat-fed hyperlipidaemic mice deficient in MPGES1 show that targeted deletion of MPGES1 results in more formation of PGI_2 compared to TXA_2 and retards atherogenesis, suggesting a favourable cardiovascular side effect profile for MPGES1 inhibitors (256, Wang, 2008 #218). Shunting of PGH_2 toward PGI_2 was also observed in a gene dose-dependent manner when mouse embryonic fibroblasts with heterozygous and homozygous deletion of MPGES1 and their wt controls were stimulated with IL-1β (263). Of particular interest is shunting of PGH_2 into PGD_2, because this can give rise to breakdown products of PGD_2 with anti-inflammatory properties, the cyclopentenones (257, 264). In such a scenario, MPGES1 inhibitors would even work as disease modifying anti-rheumatic drugs.

In contrast to targeted genetic deletion, inhibition of enzyme activity by small molecule drugs will most likely not result in the same metabolic and functional effects as seen in the studies with knock-out animals. Also, results from these studies cannot be directly transferred to the situation in humans. The difference between mice and men became dramatically clear when inhibitors with high potency in the human system failed when tested on rodent enzyme, as shown in animal experiments (176) as well as on the molecular level in paper II.

Due to the observed redirection of PGH_2 to PGI_2, the efficacy of MPGES1 inhibitors as analgesic drugs has been questioned (265). Prostanoids act as pro-nociceptive and hyperalgesic mediators. Local subcutaneous injection or intrathecal injection of PGE_2 into the spinal canal resulted in strong pro-nociceptive effects, and PGE_2 was shown to be the most prevalent prostanoid species in cerebrospinal fluid and spinal cord tissue in models of inflammatory pain (266). Similar results as with injection of PGE_2 were obtained with PGI_2, whereas results with PGD_2 and $PGF_{2\alpha}$ are ambiguous (267). Furthermore, deletion of the EP_1 (268) and the EP_2 receptors (269) or the IP receptor (270), which is activated by PGI_2, resulted in altered pain perception. Therefore, a role for pain was mainly attributed to both PGE_2 and PGI_2. On the other hand, mice deficient in MPGES1 show reduced pain perception (37), which argues for the usefulness of MPGES1 inhibitors.

Prostaglandin E_2 as a Biomarker

In order to assess possible shunting effects associated with the inhibition of MPGES1, suitable analytical techniques are necessary to detect and to quantify low concentrations of prostanoids. The biological samples used for these investigations should be easy to obtain and reflect systemic prostanoid biosynthesis. Both requirements are met by urine samples. Several LC-MS/MS methods for specific quantification of tetranor-PGEM, the major urinary metabolite of PGE_2, have been developed, however, most of these methods employ different derivatisation techniques, which makes sample preparation more laborious and time consuming, and bears the risk of unwanted chemical reactions that influence the result. A method for direct quantification of urinary tetranor-PGEM is presented in paper V.

Sample preparation of the developed method involves addition of internal standard, acidification and solid phase extraction of the hydrophobic compounds. Mass transitions for tetranor-PGEM and tetranor-PGDM as well as the respective deuterated internal standards are monitored the present state of method development, however, it can be extended to cover also the urinary metabolites of the other prostanoids, i.e. tetranor-PGFM, 11-dehydro TXB_2, and 2,3-dinor-6-keto $PGF_{1\alpha}$. Quantification is achieved with the help of an external standard curve. Samples from healthy volunteers before and after vaccination against the 2009 flu pandemic caused by influenza A virus subtype H1N1, a procedure that evokes a local inflammatory response against the injected adjuvant and antigen, were measured. An average concentration of 3.7 ± 2.1 pmol tetranor-PGEM per mg creatinine was determind, which was not significantly affected by the vaccination procedure. In a second pilot study, urine samples from children with lower respiratory tract inflammation due to infection with respiratory syncytial virus (RSV) were investigated. The levels of tetranor-PGEM were found to be 13-fold increased in the group of RSV infected children compared to samples from a group of healthy children. However, significant differences were detected in various normalisation parameters, such as such as urinary creatinine concentration, body weight and osmolality of the urine samples, which was likely due to a control group that was not well age-matched among individuals.

The results show that the LC-MS/MS method is sensitive enough to quantitatively determine basal levels of tetranor-PGEM without prior derivatisation. This is the prerequisite to assess decreases of systemic PGE_2 in response to MPGES1 inhibition. Furthermore, the tetranor-PGEM concentrations correspond to severity of inflammation and disease, as higher levels were found in individuals with RSV infection compared to healthy controls. This is in accordance with results from the literature (271) and raises the possibility of using urinary tetranor-PGEM as a biomarker of inflammation and infection.

A biomarker, or a biological marker, is defined as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (272). Applying this definition on the field of biomedicine, a very wide range of characteristics can be referred to as biomarker, including genes, gene products, proteins and enzymes, metabolites, or hormones. Even specific cells, such as leukocyte infiltrates or transformed cells that arise during carcinogenesis, complex organ functions or general characteristic changes in biological structures can serve as biomarkers. In principle, any chemical, physical or biological parameter that can be measured and interpreted in relation to the health status of a patient can be regarded as a biomarker, and in fact biomarkers have been for considerable time used in pre-clinical research and for clinical diagnosis. For example, body temperature is a well-known biomarker for fever, and C-reactive protein (CRP) is a frequently used marker for inflammation.

The crucial questions in the evaluation of a biomarker are therefore how good it is in terms of selectivity and sensitivity, at what time point during the disease progress it appears and how significant it is, and last but not least how cumbersome, laborious or painful as well as how costly it is to be assessed.

MPGES1 is up-regulated by pro-inflammatory stimuli, which results in a robust increase of PGE_2 levels associated with inflammatory diseases. Tetranor-PGEM reflects the systemic production of PGE_2 and can be accurately quantified in urine samples using the presented method. Therefore, tetranor-PGEM might constitute a useful biomarker for inflammatory diseases. However, more work remains to be done to generate further results, to validate tetranor-PGEM as a biomarker, and to evaluate the usefulness of this biomarker compared to established markers such as CRP.

Future Perspectives

The results from paper I and paper II have shed light on the structural basis for induced biosynthesis of PGE_2 during inflammation, as well as on the inhibitor binding site and the active site of MPGES1. These findings will help to unravel the precise reaction mechanism of this enzyme and facilitate future experiments using site-directed mutagenesis, steady state kinetic experiments and possibly also pre-steady state kinetic experiments. While the location of the inhibitor binding site has been identified, the precise orientation of the inhibitor remains to be investigated and the residues that interact with the inhibitor need to be identified. This problem may be addressed by co-crystallisation of the protein with the inhibitor and subsequent structural elucidation.

An increasing number of selective MPGES1 inhibitors that interfere with the enzyme activity at low nanomolar concentrations have become available for research use during the recent years. This provides a unique opportunity to characterise their beneficial effects and possible side effects in *in vitro* experiments as well as in *in vivo* animal studies using several disease models. The inhibitors may be used to further investigate the importance of MPGES1 derived PGE_2 for acute and chronic inflammation, for the perception of pain and the mechanisms behind the emergence of pain, in cancer models, as well as in other diseases, such as Alzheimer's disease.

Analytical methods, like the ones that have been used in paper IV and V, will play a preeminent role in these experiments for the quantification of locally and systemically produced prostanoids and their metabolites. This and may help to understand possible shunting effects that might occur in response to MPGES1 inhibition. The formation of anti-inflammatory cyclopentenones as a result from shunting of PGH_2 into the PGD_2 pathway has been proposed, but to date these lipid mediators have not been demonstrated *in vivo* after MPGES1 deletion or inhibition.

Finally, the use and usability of the urinary PGE_2 metabolite tetranor-PGEM as a biomarker for inflammation and infection remains to be investigated.

All of these lines of research will hopefully result in promising outcomes, so that MPGES1 inhibitors will become available in the near future and contribute to the benefit of mankind.

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