THESIS ON NATURAL AND EXACT SCIENCES B67

Prostaglandin Synthesis in the Coral *Plexaura homomalla*: Control of Prostaglandin Stereochemistry at Carbon 15 by Cyclooxygenases

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Declaration:

"Hereby I declare that this doctoral thesis, my original investigation and achievment, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any degree or examination."

LOODUS- JA TÄPPISTEADUSED B67

Prostaglandiinide süntees korallis *Plexaura homomalla*: tsüklooksügenaasi stereospetsiifilisuse struktuurilised determinandid

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CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numbers.

I Valmsen K., Järving I., Boeglin W.E., Varvas K., Koljak R., Pehk T., Brash A.R., Samel N. The origin of 15*R*-prostaglandins in the Caribbean coral *Plexaura homomalla*: Molecular cloning and expression of a novel cyclooxygenase. *Proc. Natl. Acad. S*ci. U.S.A., 2001, **98**, 7700-7705.

II Valmsen K., Boeglin W.E., Järving I., Schneider C., Varvas K., Brash A.R., Samel N. Structural and functional comparison of 15*S*- and 15*R*-specific cyclooxygenases from the Coral *Plexaura homomalla*. *Eur. J. Biochem*., 2004, **271**, 3533-3538.

III Valmsen, K.; Boeglin, W.E.; Järving, R.; Järving, I.; Varvas, K.; Brash, A.R.; Samel, N. A critical role of non-active site residues on cyclooxygenase helices 5 and 6 in the control of prostaglandin stereochemistry at carbon 15. *J. Biol. Chem*. 2007, **282**, 28157-2863.

INTRODUCTION

Cyclooxygenase (COX) enzymes catalyse the first two steps in the biosynthesis of prostaglandins (PG), thromboxanes, and prostacyclins; the conversion of arachidonic acid to PGH_2.

Prostaglandins are hormone-like molecules that participate in a variety of physiological mechanisms, therefore playing critical roles in health and disease states. The correct stereochemistry of the signal molecule is crucial for its biological activity and so is under a strict enzymatic control.

The Caribbean Sea soft coral *Plexaura homomalla* is known as the richest natural source of prostaglandins (2-5% of dry weight) and is also the only organism that contains prostaglandins with an unusual stereochemistry. Depending on the location of the coral in the Caribbean, the carbon 15 of prostaglandins is in an *S* or *R* configuration. The enzymatic basis of prostaglandin synthesis in *P. homomalla* has been studied for more than thirty years. The biochemical pathway remained unresolved largely because of the inability to develop a coral preparation capable of catalysing prostaglandin biosynthesis *in vitro*. The occurrence of prostaglandins with two different configurations only added to the mystique, and supported the idea of a non-traditional pathway in prostaglandin synthesis.

In this thesis the biosynthetic pathways to epimeric prostaglandins in Caribbean gorgonian *Plexaura homomalla* have been described and cyclooxygenases involved in catalysis cloned, functionally expressed and characterised. Using the sequence information the structural determinants of oxygenation stereospecificity at carbon 15 were determined. The main stereospecificity determinant locates in position 349 (valine in *S*-specific and isoleucine in *R*-specific COX) with the rest of the influencers being located on the same helix or its neighbour. Therefore the conclusion was made that the structural helix-turn helix motif (helices 5 and 6), which was thought to be just a central building block for proteins of the peroxidase superfamily, is involved in the control of oxygenation stereospecificity for COX-enzymes.

1. REVIEW OF THE LITERATURE

Cyclooxygenases

Cyclooxygenases (COX), also known as prostaglandin endoperoxide synthases (E.C.1.14. 99.1) are fatty acid oxidases of the myeloperoxidase superfamily. COX proteins carry out the synthesis of PGG_2 (cyclooxygenase activity) and its reduction to PGH_2 (peroxidase activity).[1,2] PGH_2 is further metabolised by tissue specific isomerases into more stable individual prostaglandins (PG), prostacyclins and thromboxanes.[3] These latter prostanoids are then able to act in a paracrine or autocrine mode through the action of G-protein-linked receptors with the subsequent generation of secondary messengers like cAMP or Ca^{2+}. Prostaglandins are found in all animal tissues where they play a role in diverse physiological and pathological processes.[4]

All vertebrates including fish, birds and mammals have two cyclooxygenases, one constitutive (COX-1) and another inducible (COX-2). Each isozyme is encoded by a different gene.[5,6] The enzymes are highly similar in structure and enzymatic activity.[7-10] The main difference between the two lies in their genetic regulation and biological roles. The COX-1 expression is constitutive in most cell types and is thought to carry out housekeeping roles in various tissues. Though COX-2 can also be constitutive in some tissues, the COX-2 expression and activity is mostly induced in response to inflammatory and proliferative stimuli.[11-13]

COX-1 and COX-2 are of particular interest because they are the major targets of nonsteroidal anti-inflammatory drugs (NSAID) including aspirin, ibuprofen and the new, selective COX-2 inhibitors. The inhibition of COX reduces inflammation, pain and fever, and with long term usage reduces the risk of cardiovascular diseases and the development of cancer and Alzheimer's disease.[14,15]

1.1. Overview of prostaglandin biosynthesis

The prostaglandin synthesis involves three phases:

- The first step is the liberation of arachidonic acid as the main precursor of prostaglandins from membrane phospholipids, usually by the action of phospholipase A_2 [16]
- Once arachidonate has been supplied, two isoforms of COX form PGH_2 via an identical enzymatic processes.
- PGH_2 is then metabolised by different enzymatic pathways to the biologically important primary prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), thromboxane A_2 (TxA_2) and prostacyclin (PGI_2).[3]

COX catalysis goes through two sequential reactions; a cyclooxygenase reaction and a peroxidase reaction [17,18] (Fig. 1). These two reactions occur at

physically and functionally separate sites and both require the presence of heme.[7,19]

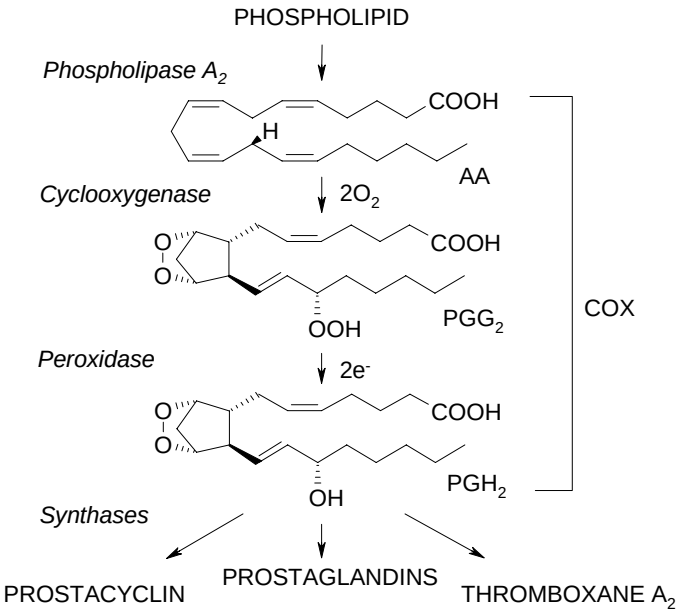


Fig. 1. There are two overall reactions catalyzed by COX proteins. The first reaction is a cyclooxygenase reaction that requires two molecules of oxygen. The end-product of this reaction is PGG_2. The second reaction is a peroxidase reaction that generates PGH_2. Subsequent cell-type specific metabolism results in the production of the primary prostaglandins, prostacyclin, and thromboxane.

The cyclooxygenase reaction involves free radical mechanism utilising a tyrosyl radical which is generated during the heme-dependent peroxidase reaction[20-22] (Fig. 2).The reaction starts when Tyr385 radical abstracts the pro-S hydrogen from the carbon 13 of the substrate. The removal of the hydrogen produces a pentadienyl radical with an electron density at carbons 11 and 15. Reaction of the radical at carbon 11 with molecular oxygen produces a peroxyl radical, which adds to carbon 9, generating a cyclic peroxide and a carbon-centered radical at carbon 8. The carbon 8 radical adds to the double bond at carbon 12, generating the bicyclic peroxide and an allylic radical with an electron density at carbons 13 and 15. The reaction of the radical at carbon -15 with molecular oxygen generates a peroxyl radical that is reduced to PGG_2.[23] The product of the reaction diffuses from the cyclooxygenase active site and binds to the peroxidase active site where its hydroperoxide group is reduced to form PGH_2.

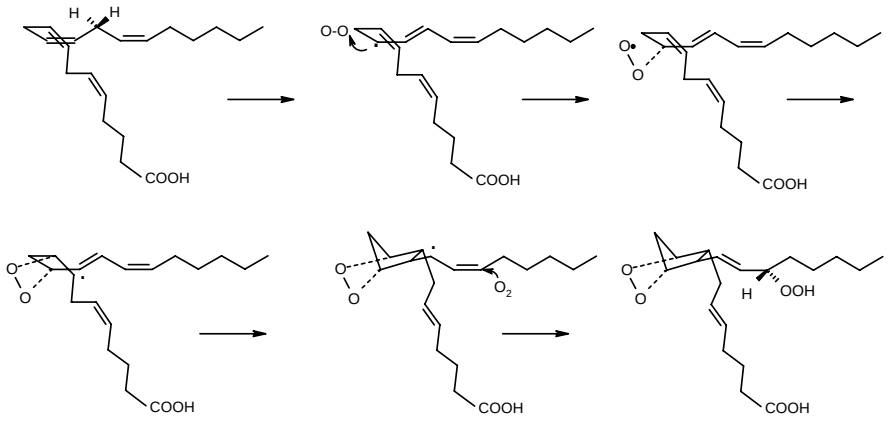


Fig. 2. A detailed proposed mechanism for the COX reaction. Abstraction of the 13-*pro*-(*S*) hydrogen atom of 20:4, followed by addition of two oxygen molecules and the formation of the cyclic peroxide ring system, leads to the formation of PGG_2. PGH_2 is formed from PGG_2 by the peroxidase activity of the enzyme.

The mechanistic relationship between the cyclooxygenase and the peroxidase activities is best described by the so-called branched-chain model.
The branched-chain mechanism predicts that any peroxide that is substrate for COX peroxidase will be an activator of the cyclooxygenase. The peroxidase reaction of COX has many similarities with other, classic peroxidases. The main intermediates of the COX reaction Compound I and Compound II are named according to the corresponding compounds from peroxidase reactions [24] (Table 1 for COX reaction intermediates). Native enzymes react with substrate peroxide (ROOH), reducing it to the corresponding alcohol (ROH) and converting the enzyme to Compound I. Two sequential one electron reductions using a peroxidase reductant (reducing co-substrate) as the electron source convert Compound I to Compound II and then back to the resting enzyme. The

Table 1

name	Description	symbol
Compound I	Two-electron oxidized state with iron as Fe^{4+} and porphyrin as cation radical	$[[PPIX^{\bullet})^{+}Fe^{4+}O]$
Compound II	One- electron oxidized state with iron as Fe^{4+}	$[[PPIX)^{+}Fe^{4+}O]$
Intermediate I	Two-electron oxidized state with iron as Fe^{4+} and protein tyrosyl radical	$[[PPIX)^{+}Fe^{4+}O]Tyr^{\bullet}$
Intermediate II	Two-electron oxidized state with iron as Fe^{4+} and unknown protein radical	$[[PPIX)^{+}Fe^{4+}O]X^{\bullet}$
Native	Iron as Fe^{3+}; no heme or protein radicals	$[[PPIX)^{+}Fe^{3+}]$

identity of the reducing co-substrate *in vivo* is not known.[25] As the COX enzyme involves two interrelated activities, then despite important similarities, the peroxidase reaction of COX is much more complex than that of other peroxidases. Compound I is converted via intramolecular electron transfer to Intermediate I, where the free radical is at Tyr385.[26] Intermediate I abstracts hydrogen from arachidonic acid or another suitable substrate, reducing the tyrosyl radical and forming a fatty acid radical (FA$^{\bullet}$). Alternatively, Intermediate I can switchover to Intermediate II, where the free radical is transferred from Tyr385 to some unknown moiety of the enzyme. Intermediate II can lead the enzyme into inactivation or, alternatively, when peroxidase reductant in stoichiometric quantities is present, the reduction of Intermediate II to Compound II.[27] Recently, Tyr504 has been identified as an alternative

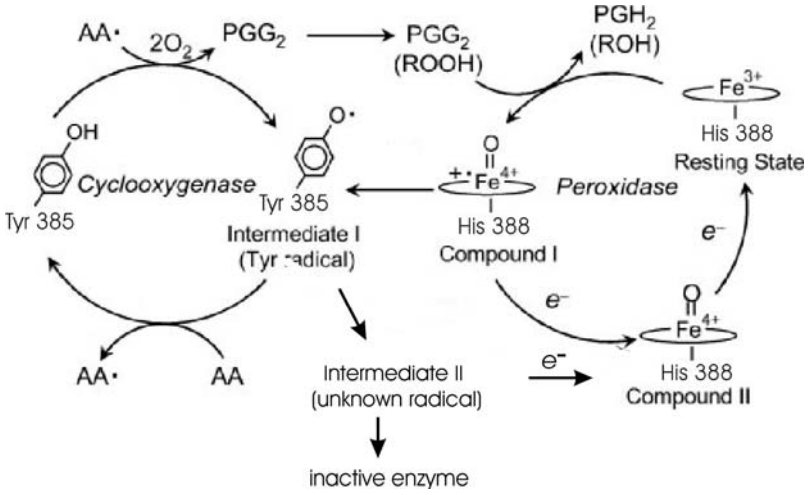


Fig. 3. A branched chain mechanism for the cyclooxygenase reaction [26] (modified from 100).The reaction starts when the heme group at the peroxidase site undergoes a two-electron oxidation by a hydroperoxide yielding Compound I which is essential for the initiation of cyclooxygenase activity.

tyrosyl radical in COX-2, being in equilibrium with Tyr385. However the radical migration from Tyr385 to Tyr504 does not significantly affect the inactivation process,[28,29] so the self-inactivation mechanism is still an unresolved issue in the cyclooxygenase catalysis.[12] It is known that there are distinct suicide inactivation processes for both cyclooxygenase and peroxidase activities. They both occur when the free radical is on a protein, on Tyr385 or on some other unknown residue.[30] Suicide inactivation is a crude regulatory mechanism which places an upper limit on cellular prostaglandin biosynthetic activity.[25]

In the branched-chain model, the hydroperoxide (PGG_2) which is produced by activated COX subsequently initiates multiple turnovers in the cyclooxygenase reaction.[26] Once the cyclooxygenase catalytic cycle is initiated, it can operate independently of the peroxidase cycle.[31] While the

cyclooxygenase initiation itself is dependent on the peroxidase turnover, the reverse is not true: peroxidase activity can occur in the absence of a functional cyclooxygenase-active site.[31,32]

The hydroperoxide level needed to activate cyclooxygenase activities differs between COX isozymes. COX-2 is activated by concentrations of hydroperoxides which are ten times lower (2 nM) than COX-1 (20 nM).[1,12,33-35] This is one mechanism that allows COX-2 to function independently of COX-1 in cells expressing both isoforms.[35,36] For that reason, COX-2 is able to be catalytically active in an environment in which much lower concentrations of hydroperoxide are available, a finding that may have physiological significance.[1,37]

Initially the heme group is probably oxidised either by an alkyl peroxide or a peroxynitrite which is derived from the condensation of nitric oxide and superoxide [38,39] (•O-O- + •N=O →O=N-O-O-).

COX-1 and COX-2 have similar cyclooxygenase turnover values (~3500 mol of arachidonate/min/mol of dimer[40,41]), and the K_m values for arachidonate (~5μM).[41,42,43]

1.2. The structural basis of the cyclooxygenase catalysis

1.2.1. The structure of COX genes

The gene that codes for COX-1 is located at human chromosome 9, which is 22 kb in length, and contains eleven exons and ten introns.[5] It is transcribed into a 2.8 kb mRNA. The gene is a housekeeping gene which lacks a TATA box.[44,5] The COX-2 gene is present on human chromosome 1, and is 6 kb in length, containing ten exons and nine introns.[45] It is transcribed into 2.8 kb and 4.6 kb mRNAs, depending on the polyadenylation site used in the 3´-untranslated region.[11,46-49] The COX-2 gene is an immediate-early gene which is induced by a variety of stimuli and contains a TATA box and several inducible enhancer elements. The COX-2 gene contains a large 3´-untranslated region which contains several evolutionary conserved regions and 22 copies of the AUUUA motifs.[50,51]

1.2.2 **The enzyme structure and mutagenesis** (the structure–function relationship)

The primary structures of COX enzymes (either directly determined or deduced from several genomic projects) have been at least partly described from more than 25 vertebrates (including mammals, birds, fish, and amphibians) and four invertebrates (coral and tunicata). Most of them contain two (COX-1 and COX-2) and certain fish even three isozymes. Curiously, a COX gene has also been found in the genome of *Cercopithecine herpesvirus*

8[52-54] but not in lower animals with fully sequenced genomes like *Caenorhabditis elegans, Drosophila melanogaster*.

COX-1 and COX-2 from the same species display a 60-65% sequence identity while the sequence identity among orthologs from different vertebrate species varies from 85-95%.[55]

The solution of the tertiary structures of COX-1 and COX-2 complexed with inhibitors helped in understanding some stereochemical aspects of the cyclooxygenase process.[7-9,56-60] Four structures of ovine COX-1 are available in complex with fatty acid bound in cyclooxygenase active site: COX-1-arachidonic acid (20:4n-6), COX-1-linoleic acid (18:2n-6), COX-1-dihomo-γ-linoleic acid (20:3n-6), and COX-1-eicosapentaenoic acid (20:5n-3).[61-63] These structures were determined using a catalytically inactive enzyme reconstituted with Co (III) protoporphyrin IX. An analogous structure of COX-2 without heme cofactor in complex with arachidonic acid is also available.[64]The structures of enzyme-substrate complexes have rendered possible the identification of the enzyme residues involved in substrate binding and positioning. Additionally, structure-guided mutagenic studies allowed the revealing of the importance of individual amino acids in cyclooxygenase catalysis, as well as the differences between isozymes which therefore yield further insights into the mechanism of prostaglandin formation and the structure-function relationship of COX enzymes (Fig. 4).

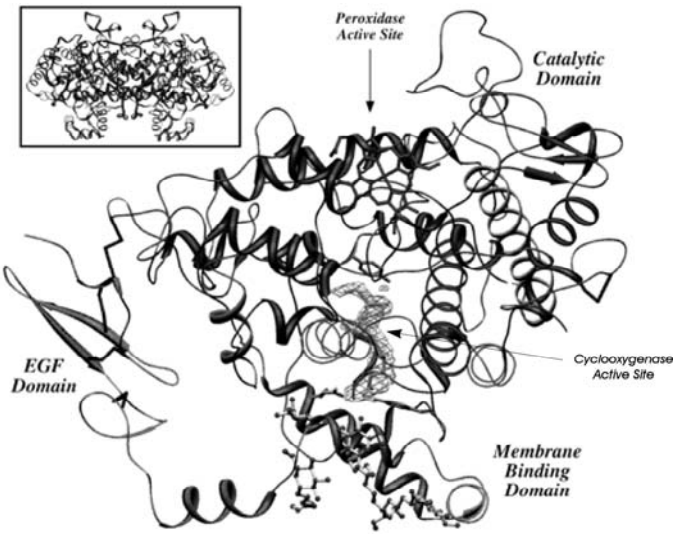


Fig 4. The tertiary structure of ovine COX-1 monomer with arachidonic acid bound in the cyclooxygenase channel. Three clearly distinguished domains: an EGF-like domain, the membrane binding domain, and the catalytic domain with distinct peroxidase and cyclooxygenase sites are shown. The inset illustrates the homodimeric nature of COX.[5]

Cyclooxygenases 1 and 2 are glycosylated homodimeric proteins found on the lumenal side of endoplasmic reticulum (ER) and on the inside of the nuclear envelope. COX proteins are termed monotopic membrane proteins, but functionally they behave like integral membrane proteins in that they require treatment with detergents to dissociate them from the membrane.[1]

Approximately 40% of the protein is α-helix and only small amount of β-sheet exists.

Although sequence homology between COX-1 and COX-2 is approximately 60%, the tertiary structure is almost identical. The root-mean-square (r.m.s) deviation between different cyclooxygenases is 0.9 Å for all the backbone carbons and is even smaller (0.4 Å) for the core catalytic residues of ovine COX-1 and human COX-2. The folding pattern of the COX monomer can be divided into three regions: the EGF-like domain, the membrane binding domain and the catalytic domain with distinct peroxidase and cyclooxygenase sites.[7-9] The main differences in COX-1 and COX-2 structure are in both N- and C-terminus.

1.2.2.1. Glycosylation

COX-1 has four potential N-linked glycosylation sites at residues 68, 104, 144, and 410, three of which are occupied. Asn 104 of the native mammalian COX-1 is not glycosylated.[65,] Glycosylation sites 68, 144, and 410 are also conserved in COX-2. COX-2 lacks glycosylation consensus sequence at 104 but has two additional glycosylation sites in the C-terminus (580 and 592 by human COX-2 numbering). When expressed in baculovirus or Cos expression system COX-2 is partially glycosylated at Asn580, but not at Asn592.[66,67] The reason for Asn592 not being glycosylated can be the presence of proline between Asn and Ser.[68] The polysaccharide structure of both endogenous and recombinant COX-1 and COX-2 expressed in both mammalian and insect cells has been found to be of a high mannose type.[67,66] The glycosylation of cyclooxygenases is required for attaining proper folding and/or oligomerisation but not for maintaining their native structures. Blocking glycosylation with tunicamycin destroys the activity of both isoforms, but glycosidase treatment of mature protein does not significantly affect activity.[69,65,41] Recently it has been shown that the glycosylation of Asn580 in COX-2 together with a whole extra region in the C-terminal is involved in targeting the enzyme to ER associated degradation (ERAD).[70]

1.2.2.2. C-terminus

The C-termini of cyclooxygenases contains tetrapeptide similar to ER retrieval signal KDEL. The terminal of COX-2 is longer than that of COX-1, containing an insert of eighteen amino acids which are six residues from the C-terminal end.[72] The C-terminal segment beyond Pro583 (seventeen amino acids

in COX-1 and 35 amino acids in COX-2) have not been resolved crystallographically.[71,9,8,7,59]

Mutagenesis studies indicated that mutations or deletions in the C-terminus did not change the subcellular localisation of recombinant COX-1 or COX-2.[73,74,34] Surprisingly, they affected enzyme activity instead, especially in the case of COX-1.[73] At the same time, the lengthening of the C-terminus did not significantly affect the activity of either isoform.[73]

These findings indicate that the extreme C-terminal region is important to the functional integrity of cyclooxygenases, but not an essential part of the intracellular targeting mechanism. The functioning of the longer C-terminus of COX-2 is distinctly more tolerant to changes than the shorter COX-1 segment.

Most recently the nineteen amino acid cassette of COX-2, with an intact Asn580 N-glycosylation site, was shown to target COX-2 for proteasomal degradation. A deletion mutation (Δ581-597) and point mutation of Asn580 glycosylation site both stabilised COX-2. Inserting the nineteen amino acid cassette of COX-2 to COX-1 destabilises the enzyme. Therefore, the C-terminal segment of COX-2 commits the enzyme to rapid degradation by an ER-associated degradation system under conditions in which COX-1 is very stable.[75]

1.2.2.3. N-terminal signal peptide

Both COX isoforms have an amino-terminal segment which is characteristic of a cleavable signal peptide, which is removed in mature protein. The length of the COX-1 signal peptide varies among vertebrates, ranging from 23 residues in humans to 26 residues in mice and 30 residues in rabbits. The sequence is 57% conserved between human and mouse. One canine COX-1 variant (misnamed as COX-3) has a thirty-residue insert in the amino-terminal signal peptide.[76]

The length of the COX-2 signal peptide is strongly conserved at seventeen residues among mammals, thus being considerably shorter than that in COX-1. Amino acids composing the COX-2 signal peptide are also less hydrophobic.[77] The sequence is 65% identical between human and mouse. No functional differences between COX-1 and COX-2 signal peptides have been reported.[78] The meaning of the signal peptide in general is not yet fully understood.[79,80]

1.2.2.4. **EGF-like domain** (residues 34-72), **disulfides and reduced cysteines.**

The EGF-like domain begins at the N-terminus of mature COX-1 and COX-2 and forms a major part of the interface between the two subunits of the enzyme. The EGF-like module is formed by two small, two-stranded β-sheets held together by three intra-domain disulphide bonds (Cys36-Cys47, Cys 41-Cys57, Cys59- Cys69) and is covalently linked to the main body of the enzyme by another disulphide bridge (Cys37-Cys159).

The primary sequence conservation in the EGF domain between the two isoforms is close to that for the whole protein. EGF-like modules have been shown to be present, in a more or less conserved form, in a large number of other, mostly animal proteins.[81,82] Their functions are unknown, but they are thought to play roles in maintaining protein-protein and/or protein-membrane[83,7] interactions.[84] The domain has also been called dimerisation domain[77], but no experimental proof is given. Anyway, as the mutation in COX-1, which disrupts one of EGF disulfide bonds, eliminates enzymatic activity,[12] the intact EGF domain is essential for protein activity.

COX-1 and COX-2 both contain one disulphide bridge outside EGF in catalytic domain (Cys569-575). Additionally COX-1 has three (positions 313, 512, and 540) and COX-2 two (positions 313 and 540) reduced cysteines. The mutation of Cys512 did not affect COX-1 activity,[85] but mutations of either Cys313 or Cys540 resulted in the loss of most COX-1[85,86] and only a part of COX-2 activity.[89] An examination of the crystal structure of COX-1 shows that Cys313 is located far from the active site, but in the most strongly conserved region of cyclooxygenases (WLREHNRV**C**). This peptide locates on a pair of helices (H5 and H6 by myeloperoxidase numbering). These helices traverse nearly the entire protein and provide an "a-frame" that is a major structural element of cyclooxygenases,[85,86] and also of other proteins of the peroxidase family.[1,87,88] In COX-2, a mutation of both Cys313 and Cys540 to serine residues decreased enzyme activity, but only by 30%.[89]

1.2.2.5.The dimer interface

A native cyclooxygenase contains two identical monomers related by a two-fold symmetry axis (non-crystallographic C-2 axis).[7] The monomer surface area buried in the dimer interface is about 13% of the total solvent-accessible surface. The EGF-like domain contributes roughly one-third of the total buried area.[59] A total of 22 intermolecular polar interactions, numerous hydrophobic interactions and bridging water molecules contribute to the packing of two monomers. Several residues are not conserved between COX-1 and COX-2. The differences have been suggested as potentially inhibiting the formation of COX-1 and COX-2 heterodimeres.[78,8.] It is also interesting to note that two of three or four polysaccharides are located near the dimer interface.[78]

The dimer interface contains a large, partially enclosed, water-filled cavity. This cavity has been proposed as an internal conduit for PGG_2 between the cyclooxygenase and peroxidase sites.[78,64]

A small channel runs from the central solvent-filled cavity through the catalytic domain and into the cyclooxygenase active site. This small channel is almost perpendicular to the main cyclooxygenase channel, which connects the active site to the membrane-binding domain.[59] It has been proposed that this channel can serve as a "release valve", allowing solvent to escape the cyclooxygenase active site when substrate enters via the membrane-binding surface.[59]

Experiments with the chimeric heterodimers of COX-2 comprising one native and one mutated monomer (R120Q or G533A), show that only one of the two sites of a dimer functions at a given time. This means that COX monomers comprising a dimer, although identical in the resting enzyme, differ from each other during catalysis. Only one monomer works. The other sub-unit may provide structural support which enables its partner monomer to function while it itself remains non-functional, not able to bind either substrate or inhibitor.[90] Despite the crosstalk between the COX sites, no cross-talk was detected between peroxidase and COX sites of adjoining monomers. The heterodimer comprised one monomer which possessed an inactive COX site (G533A) and the other, with an inactive peroxidase site (Q203R), had no COX activity, while retaining between 20-30% of peroxidase activity. The decrease in peroxidase activity (less than the predicted 50%) was proposed to be a result of the poorer interaction of monomers in the heterodimer as compared to the homodimer.[91]

1.2.2.6. The membrane binding domain (residues 74-122)

The membrane binding domain of each subunit, which follows the EGF-like module, consists of four amphipathic α-helices (helices A-D). Three of the four helices lie in roughly the same plane forming a unique monotopic membrane-binding motif where, unlike in transmembrane proteins, the polypeptide chains do not cross the phospholipid bilayer. The last helix (D) extends up from the membrane into the third, the catalytic domain. The helices also form the entrance to the cyclooxygenase channel.[92,72,84] The surfaces of the helices A, B, and C that face away from the catalytic domain are composed of hydrophobic residues providing the mechanism for attachment to one leaflet of the bilayer. It is impossible to infer exactly how deeply the proteins penetrate into the lipid bilayer,[59] but functionally they behave like integral membrane proteins and require treatment with detergents to dissociate them from the membrane.[1] The residues on the other faces of the helices are polar and may play a role in binding phospholipid headgroups.[1]

Conservation of the amino acid sequence in the membrane binding domain is rather low, with the human isoforms sharing only 33% identity in the region compared to 60% overall. The conservation rate is even lower, 24% in the residues proposed for forming the hydrophobic anchor face.[78] The estimates of the membrane binding energy, in the side chains orientated towards the putative membrane in the crystal structures,[7,9] suggest that the ovine COX-1 subunit has a stronger interaction with the membrane than does a murine COX-2 subunit.[78]

Despite the sequence differences, the secondary and tertiary structures of this domain are well conserved; 0.7 Å r.m.s deviation for all backbone atoms. The single amino acid insertion at position 106 (proline) in COX-2 does not

perturb the overall structure, but is simply incorporated into the turn between helices C and D.[8]

The helices were mutated to abolish their ability to insert into the lipid bilayer.[93] Hydrophobic residues, orientated away from the body of the enzyme and expected to integrate with the membrane lipids, were replaced by smaller neutral or hydrophilic side chains. The mutations were made separately to each helix. In each case, the mutants remained membrane-associated. Mutants of helices A and C were catalytically inactive. Mutant of helix B retained partial activity, that was resulted from unexpected but compensatory glycosylation of Asn104. When this residue was replaced with a glutamine, the mutant lost activity.[93] In native COX-1 Asn104 is not glycosylated.[65]

1.2.2.7. The catalytic domain

The catalytic domain is the largest part of the COX-protein containing the cyclooxygenase and the peroxidase activities as two spatially distinct active sites. The overall structure has direct homology to other members of the myeloperoxidase family.[83,7] The sequence homology between mammalian COX proteins and other myeloperoxidase family members is extremely low, only 14% between COX and myeloperoxidase. Nonetheless, the overall topology in the catalytic domain is strikingly similar and the two structures show greater similarity than would be predicted. The major helices involved in heme binding in both enzymes (H2, H5, H6, H8 and H12) can be superimposed with r.m.s differences in Cα positions of 1.4 Å.[7] The structural homology extends over the entire catalytic domain of COX; however the regions falling outside the catalytic domain, the membrane-binding domain and the EGF-like motif, have no equivalents in myeloperoxidase.

The catalytic domain consists mainly of helices, two of them (helices H5 and H6) generating a skeleton for the whole domain. Those two helices are the most highly conserved regions in COX proteins. A ten-residue conserved peptide in the middle of helix H5 is also present in myeloperoxidase. It has been shown that even slight changes in those helices can have dramatic effects on both cyclooxygenase and peroxidase activities.[85,86]

1.2.2.7.1. The heme- binding site and peroxidase activity

The peroxidase activity in COX has two functions: it reduces the PGG_2 produced by the cyclooxygenase step and activates the cyclooxygenase reaction. The active site is defined by the heme prosthetic group. It lies in a relatively open cleft on the protein surface, diametrically opposite the membrane binding domain.[9,7] The active site is defined primarily by helices 2, 5, 8 and 11/12.[7] Helices 8 and 11/12 are antiparallel with one another, and form one side of the cleft. The opposite side is formed by helix 2, and the floor of the cleft is formed by helix 5. The cleft is closed at one end by a loop structure comprising residues 210-222, and at the other end by a loop formed by residues

394-408.[59] The conservation of the side chain structure near the peroxidase active site is remarkably low.[78] Lysine residues in the loop (positions 211, 215 and 222) have been mutated to alanines or glutamates. Lys211 mutations were deleterious to substrate turnover, whereas Lys215 and Lys222 mutations did not much affect the activity.[94]

Comparison of the peroxidase active sites of COX enzymes with other peroxidases reveals that in COX-enzyme the site is opened to the solvent, while in other myeloperoxidase family proteins the site is buried at the bottom of a deep channel. Accordingly COX reduces a broader range of substrates (including 15-HPETE or PGG_2) than other mammalian peroxidases. Unlike with other peroxidases H_2O_2 is a relatively poor substrate for COX enzymes. Molecular dynamics simulations suggest that PGG_2 binds to the peroxidase site through a peroxyl-iron bond (α-oxygen-iron distance 2.1 Å), hydrogen bonds (the hydroperoxide hydrogen is hydrogen-bonded to His207 and β-terminal oxygen to Gln203), and van der Waals interactions involving methylene groups adjoining the carbon bearing the peroxyl group and the protoporphyrin IX. These latter interactions, which are not possible with H_2O_2, are most probably major contributors to peroxidase specificity.[91] However, when several hydrophobic residues forming a dome over the distal side of the heme of COX-enzyme were mutated into smaller amino acids no major effect on peroxidase catalysis was detected.[91]

Residues thought to be involved in the coordination of the heme iron or in peroxidase catalysis have also been examined by mutagenic analysis.[25,95-97]

The ligand proximal to the heme is **His388**, which is positioned three residues carboxyl to the end of helix 8. The H388Q and H388A mutants of COX-1 lacked both, cyclooxygenase and peroxidase activities.[96] The H388Y mutant of COX-2 retained cyclooxygenase but its peroxidase activity was diminished more than 300-fold.[98]

There is no protein ligand distal to the heme in COX. His207 and Gln203 are located approximately 5 Å above heme iron and are important in catalysis, although their functions are not fully understood.[96,99] Anyway there is no evidence for any direct interaction on the distal heme face between the heme iron and either His207 or Gln203. As the H207A mutant of COX-2 metabolised 15-HPETE to mainly 15-KETE or epoxyalcohols, then this histidine, distal to heme is predicted to play a significant role in supporting two-electron reduction.[99] The distal glutamine Gln203 has been reported to be essential for Compound I formation.[99] The glutamine residue may stabilise the developing negative charge as only a mutant that retained an amide side chain (Q203N) retained the wild-type peroxidase activity.[96,99] However, most recently the Gln203 mutations of COX were shown not to be that deleterious, and the residue was not required for the heterolytic cleavage of hydroperoxide substrates,[100,91] (heterolytic cleavage occurs through a two-electron reduction and yields the alcohol, whereas homolytic cleavage leads to number of products including ketone).

Asn382 and Thr383 are amino acids, the chain structures of which selectively influence two important aspects of cyclooxygenase catalytic regulation: activation by peroxide and the self-inactivation of COX-2.[101] Substitutions of Thr383 (histidine in most PGHS-1) with histidine or aspartate decreased cyclooxygenase activation efficiency by about 40%, with little effect on cyclooxygenase specific activity or self-inactivation. Substitutions of Asn382 with alanine, aspartate, or leucine had little effect on the cyclooxygenase specific activity or activation efficiency but almost doubled the cyclooxygenase catalytic output before self-inactivation.[101]

11.2.2.7.2. Cyclooxygenase active site (Fig. 5)

The cyclooxygenase active site is located at the top of a long hydrophobic channel (about 25 Å long),[92] extending from the membrane binding domain to the catalytic domain. The entrance to the channel, through which substrate enters directly from the lipid bilayer, is surrounded by the helices of the membrane-binding domain. The initial portion of the channel has a large volume, and is called a "lobby". The channel narrows at the top of the lobby to form a constriction comprised of Arg120, Tyr355 and Glu524. The actual cyclooxygenase active site is surrounded by helices 6 and 17 near the bottom and helices 2 and 8 at the top.

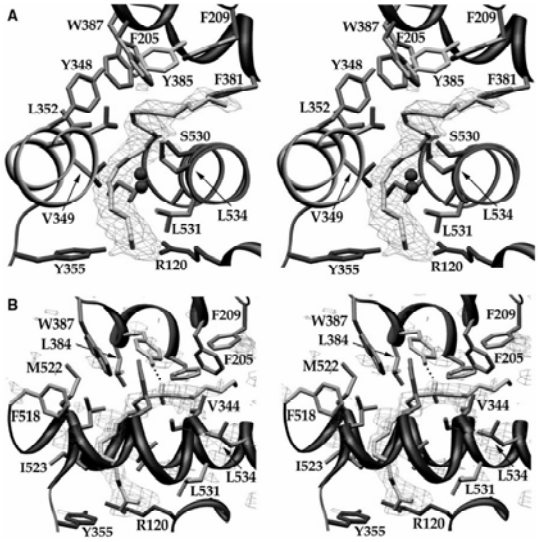


Fig. 5. Stereoview of arachidonic acid bound in the active site of COX-1. A view down helices 6 and 17. B view perpendicular to helices 6 and 17. Reproduced from ref. 1

Residues involved in hydrogen abstraction

The catalytic **Tyr385** lies in the upper part of the channel, after which the active site bends parallel to helix 17 and projects towards Gly533.[7-9,59]

The presence of **Tyr385** on cyclooxygenase active site between the heme group and substrate binding site [7-9,59] is in good accordance with biochemical evidence and the aforementioned branched-chain reaction mechanism. This residue is involved as a tyrosyl radical in abstraction of the 13-pro-*S*-hydrogen from arachidonic acid in the rate-limiting step of cyclooxygenases catalysis.[121] The tyrosyl radical connects cyclooxygenase and peroxidase activities of COX enzymes. The mutation of tyrosine to phenylalanine abolishes cyclooxygenase but not peroxidase activity.

Tyr504 is identified as alternative radical in COX-2. Tyrosyl radicals in Tyr385 and Tyr504 are in equilibrium, with the Tyr504 radical representing a reservoir of oxidant for cyclooxygenase catalysis.[28] Mutation of Tyr504 to Phe prevents radical migration thus doubling peroxidase activity.[29] However, in COX-1 the Y504F and Y504Q mutants lacked both cyclooxygenase and peroxidase activities; whereas the Y504A mutant curiously retained both activities.[97] This suggests that although the alternative tyrosyl radical is not essential for catalytic activity in either isoform, the structure of the residue can have isoform-specific effects.[28]

Tyr348 is positioned near Tyr385 in all crystal structures of COX-1 and COX-2 holoenzymes; the average distance between the phenolic oxygens is 2.8 ±0.2 Å, which is ideal for the formation of the hydrogen bond.[29] Disruption of the hydrogen bond, if it does exist,[102] by mutating tyrosine to phenylalanine has little effect on either peroxidase or cyclooxygenase activity,[102,28,121] indicating that this bonding is not important in cyclooxygenase catalysis.[102] In contrast, mutating tyrosine to leucine abolishes all cyclooxygenase and 90% of peroxidase activity. This suggests that the phenyl ring of Tyr348 is essential in positioning carbon 13 of arachidonic acid with respect to Tyr385 so that hydrogen abstraction is able to occur. When mutation of Tyr504 to phenylalanine doubled the peroxidase activity of COX-2,[28] then the introduction of another mutation (Y348F) restored the initial activity.[29] This effect also indicates the presence of hydrogen bonding between Tyr385 and Tyr348 and its role in the delocalisation of the tyrosyl radical. The addition of a Y504F mutation to Y348F mutant also partially restores loss in cyclooxygenase activity (from 50 to 80% of wt). This can be due to the radical remaining only in Tyr385, with the effect that the residue is more reactive when not hydrogen-bonded to Tyr348.[29]

Another active-site residue, which is involved in the positioning of carbon13 adjacent to Tyr-385, is **Gly533.** Gly533 is located at the end of the hydrophobic alcove 3.6Å from the ω-carbon of arachidonic acid. Mutation of this residue in COX-1 to alanine completely abolishes oxygenase but not peroxidase activity.[103,102] The homologous mutant of COX-2 retained partial oxygenase activity, but lost its ability to make 15-HETE upon acetylation. However, mutations to leucine or valine abolished oxygenase activity. When these mutants were assayed against fatty acids with fewer carbons at the ω-end, they exhibited considerable activity.[105] The results indicate that Gly533 is involved in hydrophobic interaction with arachidonic acid that is necessary to

position carbon 13 appropriately with respect to Tyr385.[102] Interestingly, mutations of other hydrophobic alcove residues in COX-1 (**Phe205, Phe209, Leu534**) had relatively modest effects on oxygenase activity and product formation,[102] whereas mutation **F381A** reduced oxygenase activity by 96% without changing the K_m for arachidonic acid.[63,102]

The constriction to the active site

The cyclooxygenase active site is separated from the opening near the membrane-binding domain by a constriction made up of residues **Arg120, Tyr355, and Glu524**.

Arg120 ion pairs or hydrogen bonds COX-enzyme to the carboxylate of the substrate fatty acid.[7,20,106-108] (Rieke JR, Smith WL *et al* proposed that Arg120 forms an ionic linkage in COX-1 and hydrogen-bonding in COX-2[106]. Mutation of this residue of COX-1 to glutamine or glutamic acid increased K_m for arachidonic acid by a thousand-fold and a hundred-fold respectively.[107,108] The same mutation on COX-2 had much less significant effects (to a factor of thirty), indicating that ionic interactions are less important for substrate binding in COX-2.[109,106] This is also consistent with the fact that COX-2 can utilise the neutral derivates of arachidonic acid (anandamide and 2-arachidonoylglycerol) as substrates.[110,111] The substitution of alanine, leucine, or phenylalanine for another constriction-site residue, **Tyr-355**, reduced oxygenase activity by a factor of five to ten and increased the K_m two to four-fold, but the magnitude of these changes was much lower than those observed on the mutation of Arg-120 in COX-1.[108,20] The changes were similar with the Y355F mutant of COX-2.[117]

Although the carboxylate group of **Glu524** appears to form a salt bridge with the guanidinium group of Arg120, mutations of this residue to aspartate, glutamine, or lysine did not significantly affect either peroxidase or cyclooxygenase activity.[108]

Although the mutations of Tyr355, and Glu524 did not affect enzyme activity, they did, as also mutations of Arg120,[107-,109] change the kinetics and selectivity of different inhibitors.[112-115]

The acetylation site of aspirin

Ser530 is the site of acetylation by aspirin[116,56,103] and is in close contact with the carbon of substrate fatty acid from where the hydrogen is abstracted (C-13 in arachidonic acid).[61,62] Ser530 of both isoenzymes have been mutated to different amino acids. [102,103,116-121] The results show that Ser530 is not important for cyclooxygenase activity, as the mutagenesis of this serine to alanine does not affect catalytic activity or arachidonate binding. This suggests that acetylation by aspirin inhibits cyclooxygenase activity by blocking the approach of the substrate to the catalytic tyrosine in the cyclooxygenase active site channel. Indeed, the substitution of bulkier side chains at position 530 inhibits the cyclooxygenase activity of both COX, whereby the substitution of

larger residues is required to inhibit COX-2 relative to COX-1.[116,103] The requirement for bulkier residues is in good accordance with the bigger size of substrate access channel of COX-2.[8]

As expected, the mutation of Ser530 to alanine eliminated the effects of aspirin on the cyclooxygenase activity of COX-1 and COX-2 preventing the acetylation of the enzyme.[117,121]

The amino acid side chain of serine also plays a critical role in determining the stereochemistry of oxygen insertion. Replacing Ser530 in human COX-2 with threonine resulted in an almost complete shift in product stereochemistry at carbon 15 towards the *R*-stereoisomer in PGG_2. Mutations to valine or methionine resulted in a partial shift of PGG_2 stereochemistry (82 or 87% respectively for human and 94% for mouse S530M). While in latter cases the main reaction product was HETE instead of prostaglandins. In human COX-1, the corresponding Ser530 mutations inactivated the enzyme.[104] This is surprising because as described before for ovine COX-1 the S530T mutant retained cyclooxygenase activity and was shown to form a significant amount of 15*R*-HETE.[103,117,113]

Residues critical for positioning arachidonic acid in a conformation so that when hydrogen abstraction does occur the substrate is appropriately arranged to yield PGG_2 (Val-349, Trp387, and Leu534).[102]

Val349 is located just above the constriction site and makes close contact with carbons 3 and 4 of arachidonic acid, which may restrict movement of the carboxyl end of the fatty acid and optimise the conformation for cyclisation.[1,61] In COX-1, the mutation of Val349 to alanine reduced oxygenase activity towards arachidonic acid by 50% but towards dihomo-γ-linolenic acid the reduction was by a factor of 800.[63] Substitution to larger residues had less pronounced effects.[102] Mutants in which Val349 was replaced with smaller residues (alanine, serine, or threonine), produced an abundance of 11-HETE versus PGG_2-derived products and little or no detectable 15-HETE; the 11-HETE / PGG_2 ratio for these mutants increased as the size of the group decreased. Replacing Val349 with a larger group (leucine) led to the generation of a relative abundance of 15-HETE (65% *S*- and 35% *R*-isomer) and no detectable 11-HETE.[122,102,63] In COX-2, the V349A mutant oxygenated arachidonic acid and dihomo-γ-linolenic acid DHLA with comparable efficiency; approximately half of those observed with a native enzyme.[63]

Val349 also has an influence on the stereochemistry of prostaglandins. When Val349 was replaced by isoleucine, the mutant COX-1 and COX-2 formed 41 and 65% 15*R*- prostaglandins, respectively. This change was highly specific for isoleucine, as mutations of Val349 to alanine, leucine, asparagine, or threonine did not alter, or only slightly altered (by $\leq$ 13%), the S-configuration at carbon 15.[104]

Trp387 sits over carbon 11 of the bound fatty acid immediately adjacent to Tyr385. This positioning should limit the conformational flexibility of the

peroxyl radical at carbon 11, thereby maximising the rate of cyclisation. As expected, a mutation of this residue to phenylalanine or tyrosine in either COX-1 or COX-2 reduces the yield of PGG_2 relative to 11-HETE.[1,123,102,62-64] The W387F variant of COX-2 produced nineteen times less prostaglandins than the wild type, while the production of 11-HETE was unchanged.[64] Interestingly, the equivalent mutation in COX-1 resulted in only a 50% reduction of cyclooxygenase activity.[123] Other substitutions had more dramatic effects. A mutation to alanine abolished cyclooxygenase and diminished peroxidase activity. A mutation to leucine nearly abolished cyclooxygenase, but even increased peroxidase activity and a mutation to arginine and serine abolished both activities.[102]

A double mutant of COX-1 **V349A/W387F** has been produced and crystallised with arachidonic acid bound to the active site.[124] The mutant mainly produces 11-HETE (>84%) with the K_m value for the oxygenation of arachidonic acid similar to that of a wild-type enzyme. Although the structure of arachidonic acid bound to the mutant is similar to that of a native enzyme, there are several significant differences. Carbons 3 to 6 of arachidonic acid are shifted into the space created by the Val349 substitution. In a native structure, Val349 contacts carbons 3 and 4.[102] In the mutant, these interactions are lost, and carbons 3 and 4 rotate and move into the space created by this mutation. Rotations in this portion of the arachidonic acid molecule results in large shifts of more rigid regions of arachidonic acid as evidenced by the significant downward shift of the C-5/C-6 double bond. There is also a difference in the location of the C-11/C-12 double bond of arachidonic acid, which is moved via rotation around the C-9/C10 bond toward the space created by the W387F mutation. In the native structure Trp387 makes two contacts with carbon 11 and one with carbon 12. The loss of these contacts contributes to a displacement of the C-11/C-12 double bond.[124]

Leu534 of COX-1 is within the van der Waals distance of carbons 15, 16, and 18 of arachidonic acid.[5] The substitution of leucine with either alanine or valine yields mutant enzymes that produce large amounts of 15*S*-HETE (56 or 47% respectively).[102] The oxygenation rate of arachidonic acid, dihomo-γ-linolenic acid, eicosapentaenoic acid and linoleic acid with L534A mutant was approximately half of that of a wild-type enzyme. The oxygenase activity of the L534V mutant was 98% with arachidonic acid, 40% with dihomo-γ-linolenic acid, and 10% with eicosapentaenoic acid. Linoleic acid was not oxidised at all by L534V.[102,62,63]

Val228 is an amino acid which resides at the junction of the main channel and the top channel of COX enzymes. When valine for COX-2 was mutated to the bulkier phenylalanine the mutant retained its wild type peroxidase activity and product profile, but upon acetylation did not make 15-HETE, suggesting that the space filled by the larger side chain is important in the production of 15-HETE.[125]

Residues comprising the active site side-pocket (523, 434, 513).

The main difference between the active sites of COX-1 and COX-2 is in the presence of an extra region called a "side pocket" in the active site of COX-2 which is not present in COX-1. The side pocket is near the base of the cyclooxygenase active site, below and across the channel from Ser530.[8] The size is determined by smaller amino acids in COX-2 in positions 523, 434 and 513; valine versus isoleucine in positions 523 and 434, and histidine versus arginine in 513 (the volume of the human COX-2 active site is calculated to be 394 $Å^3$ whereas the COX-1 site is calculated to be 316 $Å^3$). This extra volume is a structural feature exploited by most COX-2 specific inhibitors. It is also the basis for the different response by isozymes to aspirin inhibition. The side pocket region provides the critical space necessary to allow the binding of arachidonic acid in aspirin-treated COX-2 so that 15-HETE formation can occur.[125] These three amino acids in COX-2 have been mutated to the corresponding residues in COX-1. The triple mutant **V434I/R513H/V523I** had cyclooxygenase and peroxidase activities comparable to those of wild-type COX-2. However the mutations completely eliminated the ability of acetylated enzyme to generate 15-HETE.[125] When each of the three residues was individually mutated, only partial elimination of 15-HETE synthesis was achieved. The greatest reduction was seen with the **V523I** mutant.[125]

Adversative mutants were also created where COX-2 specific amino acids were introduced into the active site of COX-1. The residues Ile523 and His513 were individually mutated into valine and arginine, respectively, and the double mutant (H523R/I523Vl) was constructed. These mutations had no effect on catalysis but the mutants were sensitive to time-dependent inhibition by selective COX-2 inhibitors.[126]

Surprisingly, in zebrafish both COX-1 and COX-2 have Val and Arg in positions 523 and 513 respectively, and despite that the COX-2 selective inhibitor NS-398 maintained selectivity towards the COX-2 of zebrafish.[127] Several other non-mammalian vertebrates (other fish,[128] chicken,[46] and two different frog types,[129]) deviate from the opened side pocket rule for COX-2, so the rule appears to be valid only in respect of mammalian COX. Consequently, amino acid changes which have been thought to contribute to the structure which determined the division to isozymes are not decisive.

Other active site residues that have been investigated in regard to the contribution to catalysis

Both COX isoforms contain Leu at position **531**. In COX-2, Leu is positioned directly across from the side pocket in the cyclooxygenase active site.[8,9,64] The substitution of Leu531 in COX-1 with similarly sized hydrophilic residues (aspartic acid and asparagine), served to decrease the V_{max} values for arachidonic acid but had little effect on the K_m values.[102,103] In contrast, substitution with smaller residue (alanine) caused a 25-fold increase in the K_m.

In the COX-2 mutations of Leu531 to isoleucine, valine and alanine significantly decreased the oxygenation rate of arachidonic acid.[130]

Leu352 from COX-2 was substituted with alanine, valine, isoleucine, methionine, or glutamine. The mutations had almost no effect, either on activity or on the profile of products.[131]

Ser353 makes contact with carbon 3 of arachidonic acid at the side which is opposite to the carbons 3 and 4 contacts made by Val349. When this residue of COX-1 was mutated into glycine, alanine, or threonine, all of the mutants retained most of the activity. A slight change in the product spectrum was determined with S353T, which started to form larger amounts of both 11-HETE and 15-HETE.[102] A double mutant V349A/S353T, designed to remove all contacts between carbons 3 and 4, had only 3% of the cyclooxygenase activity of the native enzyme as compared to the single mutants that had 55% (V349A) and 42% (S353T) of activity. That points out the importance of contacts made by carbon 3 of arachidonic acid.[102]

Ile377 lies at the distal end of the active site and makes contact with the ω-end of arachidonic acid. The substitution of isoleucine with valine in COX-1 did not overmuch change the enzyme activity, but the mutant started to make somewhat more 11-HETE.[102]

Phe518, Met522, and Leu384 are outside the van der Waals contact distance for arachidonic acid in COX-1 crystal structure,[61] but have contacts with active site residues, therefore forming part of the first shell of the cyclooxygenase channel.[61]

Met522 of COX-1 was mutated to alanine, leucine, and isoleucine and no big differences from wild-type enzyme were detected.[102]

Phe518 of COX-1 was mutated to alanine, leucine, methionine, and tyrosine,[102] and of COX-2 it was mutated to isoleucine, phenylalanine, and tryptophan.[131] All mutants retained most of their activity. The production of HETEs slightly increased relative to prostaglandins.[102,131]

Substitutions of **Leu384** with alanine, valine, or phenylalanine had no effect on the distribution of oxygenated products in COX-1.[102] However in COX-2 L384W and L384F mutants started to form novel products diepoxyalcohols.[131] The same products were also formed by **Gly526** mutants (G526A, G526S, G526T, and G526V) of COX-2.[131]

Arg277-loop

The catalytic domain also contains the major structural landmark Arg277 loop, which is proteolytically sensitive in COX-1 but not in COX-2.[78] COX-2 has a trypsin-resistant proline residue in this position. Interestingly a replacement of proline with arginine (P277R; insertion of potential trypsin site) in COX-2 did not change the enzyme protease sensitive.[132]

1.3. Cyclooxygenase Substrate Specificity

Arachidonic acid is the preferred fatty acid substrate for both COX-1 and COX-2. However, *in vitro* both enzymes also oxygenate various n-3 and n-6

C_{18} and C_{20} fatty acids with catalytic efficiencies in the range of 0.05-0.7 of that in respect of arachidonate. The prerequisite for endoperoxide formation is a presence of at least three methylene-interrupted *cis*-double bonds.[17,25] The metabolism of 20:3, 20:4, and 20:5 yields PGG_1, PGG_2, and PGG_3 respectively with relative efficiencies (20:4> 20:3>> 20:5).[133] C_{18} fatty acids are oxidised to monohydroxy products. The ability of COX-2 to oxygenate alternative substrates is somewhat better than of COX-1. Crystallographic data for catalytically inactive COX complexed with different fatty acids,[61-64] and mutagenesis analysis,[110,130,115] allowed the identification of some structural elements of the discrimination between COX isozymes. There are two main reasons for that: the size of the cyclooxygenase active site and differences in the way of substrate binding (COX-1 relies mainly on ionic interactions whereas COX-2 uses H-bonding to bind the substrate). That is why, in contrast to COX-1, COX-2 has the ability to metabolise neutral substrates endocannabinoids, arachidonoylethanolamide (AEA, anandamide) and 2-arachidonoylglycerol (2-AG),[111,134] and N-arachidonoylglycine (NAGly).[115] They are metabolised into corresponding endoperoxides and hydroxy acids with a stereochemistry identical to the stereochemistry of arachidonic acid derived products.[134-136,115] The physiological significance of these findings is unclear because the K_m of human COX-2 for AEA is two orders of magnitude higher than that of the tissue concentration.[137]. However the concentration of 2-AG reported in several tissues is in the same range as K_m.[134]

The structural basis for the ability of COX-2 to oxidise these arachidonic acid derivatives has been explored by site directed mutagenesis.[110,130,115] Amino acid mutations that are deleterious in arachidonic acid oxygenation (Y385F, G533V) have the same effect in those alternative substrates' oxygenation.

R120Q and E524L mutations reduced oxygenation rates for all three substrates, especially for 2-AG and NAGly. Y355F mutation had little effect on either of them.

Amino acids in COX-2, representing conserved differences between the active sites of the two isoforms, were mutated into their corresponding COX-1 residues both individually and together. The triple mutation (V523I/R513H/V434I) in COX-2 reduced the oxygenation of AEA, 2-AG, and N-arachidonoylglycine by 80%. The main determinant was Arg513, which sits at the base of the side pocket opposite to Arg120.[110,130,115] Arg513 was proposed to H-bond the amide or ester derivatives of arachidonic acid.[110]

The conservation of the side pocket in all COX-2 proteins suggests that the pocket was developed as part of the evolutionary process simply to confer novel substrate selectivity to COX-2 and that the physiological function of COX-2 is to oxygenate neutral substrates. Therefore, the ability of COX-2 to oxygenate neutral substrates may be the reason why the second isoform of cyclooxygenase was evolved.

PGG_2 is considered to be a natural substrate for peroxidase activity of COX, but other organic peroxides including primary and secondary hydroperoxides

and, to a lesser extent, tertiary hydroperoxides, can also be reduced. H_2O_2 is relatively poor substrate for COX enzymes.[1]

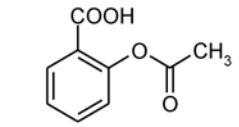
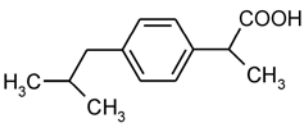
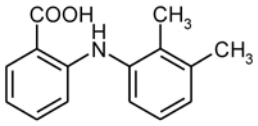
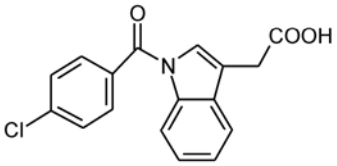
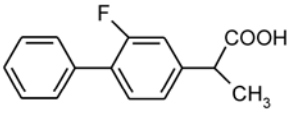
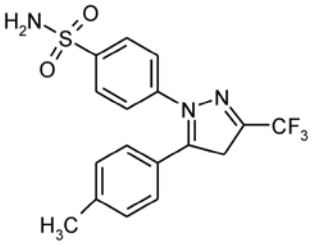
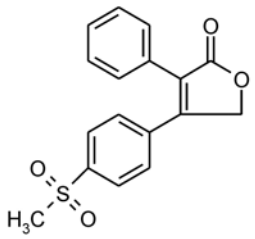
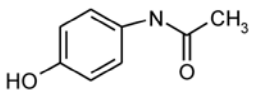
1.4. The inhibition of COX proteins by Nonsteroidal Anti-inflammatory Drugs

Humans have been using nonsteroidal anti-inflammatory drugs (NSAID) in various forms for more than 3,500 years,[138] and they are still our favourite medicine.

The therapeutic efficacy of NSAID derives from their ability to inhibit COX enzymes.[139] Among the NSAID in clinical use, only aspirin and the most recently discovered aspirin derivates are covalent modifiers of COX. Other traditional NSAID inhibit enzymes by competing with arachidonic acid for binding in the COX site. They differ from each other in whether they bind in a cyclooxygenase active site in a time-dependent or independent fashion,[9] and a further difference is their selectivity towards either COX-1 or COX-2.

Aspirin prevents substrate access to an active site by covalently modifying serine residue near the active site.[116] However, while acetylation of Ser530 of COX-1 completely abolishes the cyclooxygenase activity, acetylated COX-2 retains activity to convert arachidonic acid to 15*R*-HETE.[116,140] The biological role of 15*R*-HETE, which differs from the lipoxygenase-derived 15*S*-HETE in stereochemical configuration, is not completely understood. 15*R*-HETE can be converted by lipoxygenase isoforms into 15(R)epi-lipoxinA_4 also known as "aspirin-triggered lipoxin" (ATL).[141] ATL exerts potent anti-inflammatory activities and inhibits polymorphonuclear granulocyte requirement at the sites of inflammation.[142,143]

Traditional non-selective NSAID have proven highly effective and safe in short-term management, while long-term studies demonstrated serious gastrointestinal toxicities. It is currently hypothesised that the undesirable side-effects of NSAIDs are due to COX-1 inhibition, whereas the beneficial effects, such as the reduction of swelling and analgesia, are related to COX-2 inhibition. Therefore, highly selective COX-2 inhibitors including celecoxib, rofecoxib, valdecoxib, lumiracoxib and etoricoxib were developed with the hope of significantly reducing the side effects associated with chronic high-dose NSAID use. The number of COX-2 selective inhibitors developed had already exceeded 500 by 2001,[144] which is just ten years after the discovery of the existence of the second isoform. The extra volume in the cyclooxygenase active site is the main structural feature exploited by most COX-2 selective inhibitors.[55] The other structural difference between COX isoforms that determines difference in response to inhibitors is the interaction between

 Acetyl salicylic acid (Aspirin)	Covalent modifier. In case of low doses is selective COX-1 and in high doses nonselective inhibitor
 Ibuprofen  Mefenamic acid	Reversible, competitive nonselective inhibitors. Show no time dependence. They inhibit COX activity instantaneously and are readily washed out when NSAID is removed.
 Indomethacin  Flurbiprofen	Slow, time-dependent, reversible nonselective inhibitors. Require seconds to minutes to bind the active site. Once bound, however, these drugs have low off-rates that may require hours to wash out of the active site.
 Celecoxib (Celebrex)  Rofecoxib (Vioxx)	Time dependent, irreversible first generation COX-2 selective inhibitors. They are weak time-independent competitive inhibitors of COX-1, but potent time-dependent inhibitors of COX-2.
 Acetaminophen (Paracetamol)	Nonselective inhibitor with mechanism that is still not completely understood.

carboxylic acids and the active site residue Arg120. For this reason, by neutralising the acidic NSAID, molecules are generated which retain the ability to bind to COX-2 but not to COX-1.[145]All the first and second generation COX-2 inhibitors are diarylheterocycles, but structurally divergent compounds are under development.[145] In addition, several strategies have been described for re-engineering non-selective NSAID into selective COX-2 inhibitors.[119]

In September 2004 rofecoxib (a first generation COX-2 inhibitor) was withdrawn from the market because results from a placebo-controlled trial

suggested that its use was associated with cardiovascular events (including myocardial infarction, cardiac arrest, stroke, and pulmonary embolism). Currently it is unsure whether the hazard extends to all coxibs or is an exception. Despite all the investigations which have been carried out, it is still not clear through which mechanism COX-2 inhibitors affect the cardiovascular system. One possible explanation is that upsetting balance between two prostanoids: COX-2 derived prostacyclin (PGI_2) and COX-1 derived platelet thromboxane (TxA_2) was likely to be the mechanism of cardiovascular effects.[146] (Namely COX-1 is the only COX expressed in platelets, and PGI_2 - a dominant product of endothelium - is under physiological conditions derived largely from COX-2.)
There have been calls for the removal of all COX-2 selective drugs from the market and a return back to traditional NSAID. At the same time, it is possible that the cardiovascular risks are connected to NSAID in general and not just COX-2-specific inhibitors. It could simply be that the traditional NSAID did not have to pass the clinical standards required nowadays, and unfortunately it is difficult to find sponsors for the trials of generic agents.[146] Maybe we have to admit that just as low-dose aspirin is effective in the prevention of myocardial infarction and stroke and causes a small but definite risk of serious gastrointestinal adverse effects, so long-term usage of the selective inhibitors of COX-2 relieve pain and inflammation and convey a small but definite risk of myocardial infarction and stroke.

Acetaminophen (ApAP) is one of the most commonly taken drugs to treat fever and pain. The analgesic and anti-pyretic benefits of ApAP are due to its unique ability to selectively reduce COX products in the central and/or peripheral nervous system.[147] ApAP differs from other NSAID and selective COX-2 inhibitors because it lacks significant anti-inflammatory activity. Moreover as opposed to aspirin, ApAP is a poor inhibitor of platelet function at doses that are antipyretic.
Experimental data about the ability of ApAP to inhibit COX-enzymes is controversial. In experiments with broken cells, ApAP does not inhibit COX enzymes and according to several studies it even activates them.[148]
Experiments with intact cells showed that ApAP is a very potent inhibitor of COX-2[149]. Purified COX enzymes were both sensitive to ApAP inhibition.[148,150] Because of all the discrepancies, the mechanism of the action of ApAP is still debated.
Investigations of the mechanism of the action of ApAP against COX have focused on different topics. For the last few years the most popular of these has been the theory of a third ApAP sensitive COX isoform, so called COX-3.[76] This enzyme is the product of an alternatively spliced mRNA of COX-1 gene that is identical to COX-1 mRNA except that intron 1 is retained. However "COX-3" is unlikely to have prostaglandin –producing activity in human tissues because the retention of intron 1 in COX-1 would actually shift the "COX-3" sequence out of frame, resulting in a truncated, inactive protein.[151,152]

Indeed, most recently, "COX-3" from a rat,[153] a mouse,[154] and a human,[155] has been expressed and demonstrated to have no COX activity. So it seems much likely that "COX-3" cannot be the target of acetaminophen.[156-158]

The second popular solution to the ApAP mystery is that, differently from other NSAID, it does not inhibit COX by blocking the cyclooxygenase active site but instead blocks activity by reducing the higher oxidative state of COX peroxidase to the ferric or resting state.[148-150,159] A known aspect of prostaglandin biosynthesis is that COX enzyme needs a reducing co-substrate for its peroxidase activity. Reducing agents such as phenol have dual effects on COX activity. In lower concentrations they stimulate the enzyme while in higher concentrations they inhibit it.[148,160] ApAP, which is also a phenolic compound, appears to inhibit COX activity through the same mechanism; by its capacity to serve as a reducing co-substrate for peroxidase active site and therefore reducing the active oxidised form of the enzyme to an inactive, resting form.

ApAP keeps COX in a resting state when the concentration of peroxide is low.[159] This also explains why the central nervous system is especially sensitive to ApAP inhibition. Specifically, nerves, which are very sensitive to intracellular oxidants, actively reduce the intracellular oxidation state.[149]

1.5. Prostaglandins in Invertebrates

Prostaglandins or prostaglandin-like compounds have been reported to be present in various invertebrate species. Although there is usually detailed understanding of the actions of these compounds, knowledge of the mechanism of their biosynthesis is often lacking.

Prostaglandins exert many actions in insect biology, including ion transport, protein trafficking, reproduction, and immunity.[161] Prostaglandins in insects are proposed to be products of the COX pathway,[161] although attempts to clone functional COX enzyme from several insects have not been successful (Varvas private communication). Furthermore, *Caenorhabditis elegans* and *Drosophila melanogaster* have been fully sequenced and they do not contain sequences similar to COX genes. However, there have been signs of the presence of PGE_2 receptors in tobacco hornworms and *D. melanogaster*.[162] PGE_2 receptors have also been cloned from anthropods,[165] and most recently the cloning of a functional prostaglandin-synthesising enzyme similar to ordinary COX from two different anthropods has been successful (Varvas unpublished results).

Ciona intestinalis is a member of the Phylum Chordata that includes the vertebrates, and urochordates (sea squirt, salps and cephalochordates). Recently draft genomes of *C. intestinalis* and *Ciona savignyi* have been published.[163] The sequence studies,[164] revealed that the sea squirt possesses two COX genes with a 50% intraspecies identity.

There is no evidence for the presence of COX genes in unicellular species or sponge.

1.5.1. Prostaglandins in Coral

1.5.1.1 The Gorgonian *Plexaura homomalla*

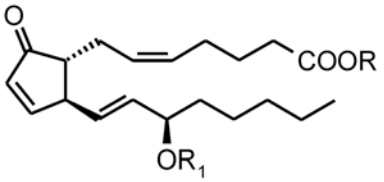
Prostaglandins were first discovered in a marine life form in the Caribbean gorgonian *Plexaura homomalla.*[166] From that time *P. homomalla* has been under an intense level of attention because of its unique composition of polyenoic acid metabolites.[167] On account of the huge prostaglandin content, *P. homomalla* served as a source of prostaglandins for research until an efficient chemical synthesis was developed.[168]

Gorgonians or horny corals are members of a zoological group which comprises around one thousand species. They are large, and some species are brightly coloured.[169] *P. homomalla* is one of the most common horny corals and it belongs to the class Octocorallia, order Gorgonacea, Plexauriidae.[169]

1.5.1.2 *Plexaura homomalla* natural products

P. homomalla is known as the most abundant natural source of prostaglandins, containing up to 5% of its dry weight prostaglandins and their derivates.[167,83,170] The most abundant prostaglandins found are mono- and bis-ester of 15*R*-PGA_2[171,172] (3,2). The free acid of PGA_2 (1) has been found in smaller quantities. The content of esterification depends on the way corals are handled. It was shown that if the Gorgonian is handled with extreme care (frozen with liquid N_2 immediately after getting to the surface and then extracted with organic solvents), only the bis-ester of PGA_2 is isolated.[167] In addition to the prostaglandins of the PGA_2 group, an analogous series of free acids and esters from PGE_2 have also been isolated,[171] (4-6). Besides prostanoids in 15*R* configuration the same series of PGA_2 and PGE_2 of 15*S*-isoforms were isolated (7-10). Additionally, 15*R*-PGB_2 (11) and 15*S*-PGB_2[167] (12) were isolated. It appears that the corals' ability to make 15*R* or 15*S* prostanoids correlates with the geographical region of their collection.[173] *P. homomalla* collected from the Florida Keys contains 15*R* isomers of prostaglandins. When the same specimen was collected from other locations such as the Cayman Islands and the Bahamas the prostaglandins were with the 15*S*-hydroxy group . There are also reports of individual colonies that contain both 15*R*- and 15*S*-isomers in approximately equal amounts.[171]

The large scale collections of *P. homomalla* also reveals minor quantities of different prostaglandin derivatives such as 15*S*-$PGF_{2\alpha}$ (13), 5-*trans*-15*S*-PGA_2 (14), 13-*cis*-15S-acetoxy-PGA_2, (15), 13,14-dihydro-PGA_2 acetate, (16) 9-O-acetate of $PGF_{2\alpha}$ methyl ester (17) and 11-O-acetate of $PGF_{2\alpha}$ methyl ester (18).[167]



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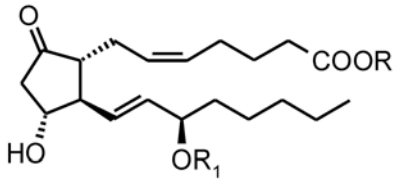
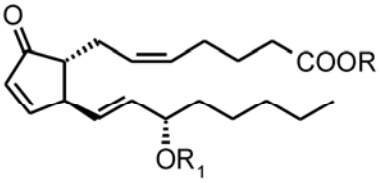
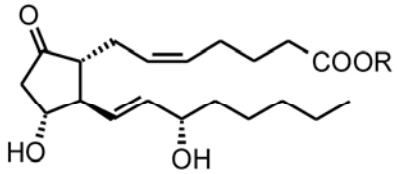
2) R=CH_3, R_1=Ac
3) R=CH_3, R_1=H



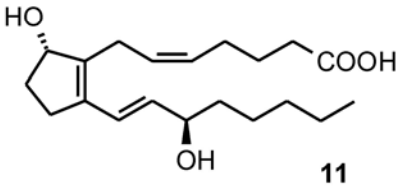
4) R=H, R_1=H
5) R=CH_3, R_1=H
6) R=CH_3, R_1=Ac



7) R=H, R_1=H
8) R=CH_3, R_1=H



9) R=H
10) R=CH_3



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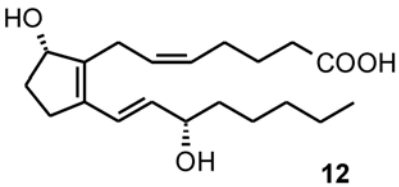

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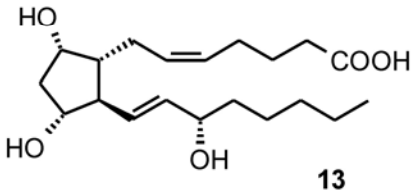

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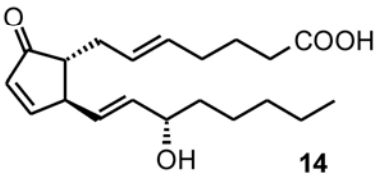

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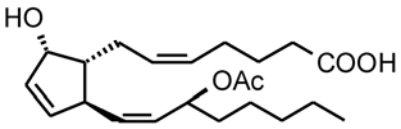

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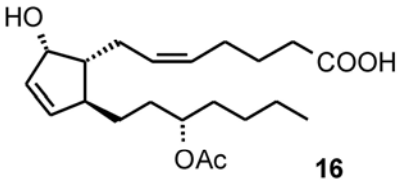

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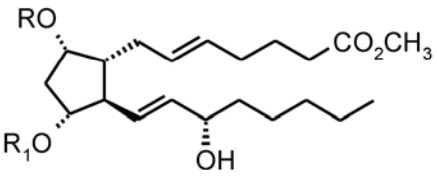


17) R=Ac, R_1=H
18) R=H, R_1=Ac

Several possible roles for the prostaglandins in *P. homomalla* have been suggested, including the regulation of ion and/or water transport and a defence against predation or parasitism.[172] It has been shown that both 15*R*- and 15*S*-PGA_2, given orally, produced vomiting in fish, and that these fish quickly learned to avoid food which contained either isomer. So the role of prostaglandin production by *P. homomalla* seems to be involved in the chemical defence of these soft bodied organisms against predation by fish, and perhaps invertebrates as well.[172]

1.5.1.3. Prostaglandin biosynthesis in *Plexaura homomalla*

The biosynthesis of prostaglandin derivatives by *P. homomalla* has been the subject of intense investigations for about thirty years. Despite the high content of prostaglandins, however, the biosynthetic pathway of these compounds has remained unresolved.[174-176] Early investigations demonstrated that the coral utilises a route other than the cyclooxygenase pathway typical of mammalian prostaglandin biosynthesis. It was shown that coral preparations were unable to convert exogenous PGH_2 or PGG_2 (endoperoxide intermediates in the mammalian COX route) to prostaglandin products.[167] Subsequently, in the work with *P. homomalla* as well as through parallel investigations with several other species of coral, a pathway has emerged beginning with arachidonic acid and its initial conversion to 8*R*-HPETE by an 8*R*-lipoxygenase, dehydration to an allene oxide, and then cyclisation to carbocyclic oxylipins,[170,177,178] (Fig. 6, righthand side). This is clearly related to the pathway in higher plants which leads to jasmonic acid,[179,180](Fig. 6, lefthand side). The experimental proof of this hypothesis has been hampered by an inability to develop a coral preparation that is able to perform the entire biosynthetic pathway beginning with arachidonic acid and finishing with optically active prostaglandins. Cell-free preparations of *P. homomalla* metabolise exogenous arachidonic acid into 8*R*-HPETE, a mixture of 8*S*- and 8*R*-hydroxy-9-oxoeicosa-5*Z*,11*Z*,14*Z*-trienoic acid (α-ketols) and racemic mixture of 9-oxo-(8,12-*cis*)-prosta-5*Z*,11*Z*,14*Z*-trienoic acid (preclavulone).[177,170] The same products are also produced by a recombinant fusion protein cloned from *P. homomalla*. This unique protein consists of a C-terminal 8*R*-LOX domain and an N-terminal heme-containing AOS domain.[178] The 8*R*-LOX, different from earlier cloned isozymes from the same coral, is similar to mammalian lipoxygenases in size, sequence and substrate specificity. The AOS domain uses the 8*R*–hydroperoxide from the same enzyme as its preferred substrate.[181] Several AOS enzymes have been cloned from different plants. They all belong to the hydroperoxide metabolising cytochrome P450 family. The coral AOS does not exhibit sequence homology to the plant AOS, but has an 11% identity to catalase. Recently, after crystallisation, it turned out that the enzyme also has a striking structural homology with catalases. However, coral AOS cannot catalyze the dismutation of H_2O_2 to water and oxygen molecules, in other words, the catalase reaction.[182]

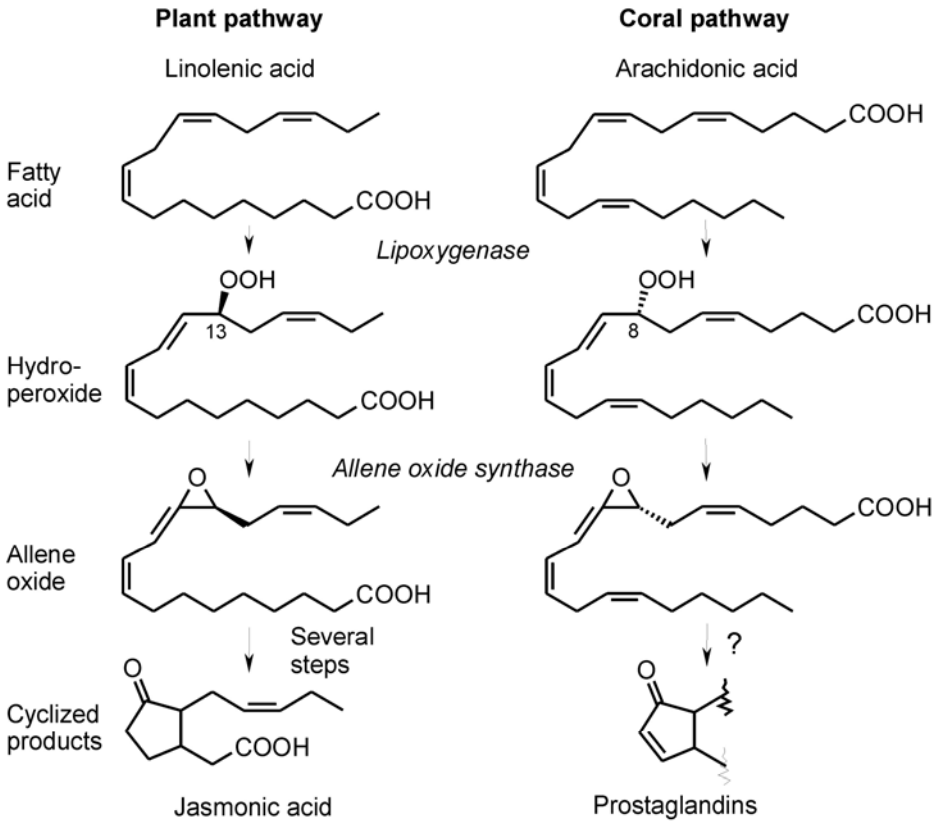


Fig. 6. Allene oxide biosynthesis and methabolism in corals and plants.

It is notable that none of the above biosynthetically produced products was detected in coral extracts in spite of the occurrence of the substrate arachidonic acid and a very active 8*R*-lipoxygenase and allene oxide synthase. Therefore the question of how gorgonians biosynthesise optically active prostaglandins has remained unresolved. This issue will be discussed in detail in the Results and Discussion part of the current doctoral thesis. We demonstrated that without a doubt prostaglandins in coral are formed from arachidonic acid via a conventional cyclooxygenase route. But still a curious contradiction is apparent between arachidonic acid metabolism *in vivo* and *in vitro*. Despite an effective cyclooxygenase route *in vivo*, coral preparations utilise lipoxygenase allene oxide synthase.

1.5.1.4. Prostaglandin biosynthesis in *Gersemia fruticosa* [183-186]

Earlier investigations in our laboratory have shown that unlike all the other corals studied previously, the White Sea soft coral *G. fruticosa,* has the ability to biosynthesise optically active prostaglandins *in vitro* conditions. The acetone powder of *G. fruticosa* converts exogenous arachidonic acid into typical mammalian prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$ and 15-keto- $PGF_{2\alpha}$. The same four prostaglandins obtained above are all present in the coral extracts as free acids. It has been shown that the prostaglandin biosynthesis in this coral occurs via the prostaglandin endoperoxide pathway. The prostaglandin intermediate, PGG_2, was isolated from short incubations. Unlike mammalian COX, coral COX has a very low peroxidase activity, and PGG_2 is accumulated instead of PGH_2. The further conversion of PGG_2 to prostaglandin final products occurs mainly non-enzymatically.

It has been found recently that, as with *P. homalla,* an 8*R*-lipoxygenase/allene oxide synthase pathway exists in *G. fruticosa*. An 8*R*-lipoxygenase/allene oxide synthase pathway led to the formation of 8*R*-HPETE and α-ketol and is not involved in the formation of prostaglandins (Lõhelaid unpublished results).

2. AIMS OF THE PRESENT STUDY:

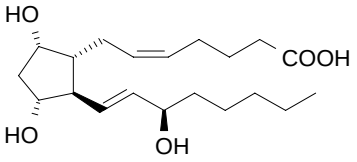
The main aim of the current study was to elucidate the pathway of prostaglandin biosynthesises in the coral *Plexaura homomalla* and to figure out the reason for the presence of epimeric prostaglandins in the two variants of coral.

Consequently the specific aims were:

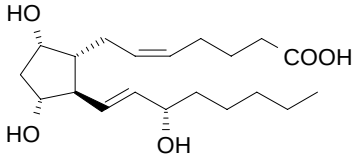
1. To identify the enzymes responsible for the synthesis of prostaglandin stereoisomers in the coral *P. homomalla*
2. To clone, express, and characterise these enzymes
3. To elicit the structural determinants of oxygenation stereospecificity at the carbon 15 of prostaglandins

RESULTS AND DISCUSSION

The Caribbean gorgonian *Plexaura homomalla* contains levels of prostaglandin which are several orders of magnitude higher than those of other animals. Depending on the geographical location, the coral is known to contain either typical mammalian 15*S* or the unusual 15*R*- prostaglandins.[187,173]



15*R*-$PGF_{2\alpha}$



$PGF_{2\alpha}$

The enzymatic basis of prostaglandin synthesis in *P. homomalla* has been studied for more than thirty years. The biochemical pathway remained unresolved largely because of the inability to develop a coral preparation capable of catalyzing prostaglandin biosynthesis *in vitro*. The occurrence of prostaglandins with two different configurations only added to the mystique, and supported the idea of a non-traditional pathway in prostaglandin synthesis.

Earlier experiments in our laboratory indicated the possibility that a COX-like enzyme may be present in the White Sea soft coral *Gersemia fruticosa.* That coral has the ability to biosynthesise prostaglandins *in vitro,* and the intermediate of cyclooxygenase route, a prostaglandin hydroperoxy endoperoxide (PGG_2), has been isolated.[185]

3.1 Molecular cloning (Articles I and II)

During the present study using a homology-based reverse transcriptase-PCR strategy, two different cDNA corresponding to COX proteins from two different samples of *P. homomalla* were cloned. One sample was from the Bahamas with the endogenous content of ordinary 15*S*-prostaglandins, and the other sample was collected from the Florida Keys, containing prostaglandins with 15*R*-configuration (GenBank accession numbers: AY004223 for 15*R*-COX and AY615733 for 15*S*-COX. The cloning strategy was based on the design of degenerate primers representing the amino acid sequences of conserved regions of mammalian COX-isozymes. The same set of degenerate primers was used before, in cloning COX cDNA from Arctic coral *G. fruticosa*.[186] According to the sequence of the first clone, gene-specific primers were designed and the COX cDNAs were PCR cloned from the coral preparations.

The ORF of 1776 bp encodes proteins (592 aa) with a 97% amino acid identity having a difference in seventeen amino acids (Fig. 7). The identity with mammalian COX-1 and COX-2 was approximately 50% to each of them.

Fig. 7. Sequences of 15*S* and 15*R*-specific cyclooxygenases in *P. homomalla*. Amino acid differences between the enzymes are boxed. Some of the functionally important residues are shown above the sequences in ovine COX-1 numeration. The different domains: a signal peptide (1-23), the EGF-like domain (24-63), a membrane-binding domain (64-107), and a catalytical domain (107-592), are separated by vertical bars.

(The alignment with several mammalian and vertebrate COX-enzymes is presented in the Appendix II.)
The presence of COX protein(s) has been shown in a diverse range of animals from fish to mammals. The question of at what stage of evolution did the different isozymes, designated currently as COX-1 and COX-2, appear remains unanswered, however. The presence of two COX-enzymes has been described in mammals, birds, and amphibians. Things become more complicated with fish. A bony fish has been shown to have both isozymes,[128,188] with a strong sequence identity to their mammalian counterparts. However, until now, only one isoform has been cloned from more primitive fish, the shark *Squalus acanthias*. This COX isoform has some features of both COX-1 and COX-2, yet it is more similar to COX-1.[189] The N-terminus is similar to COX-2 and

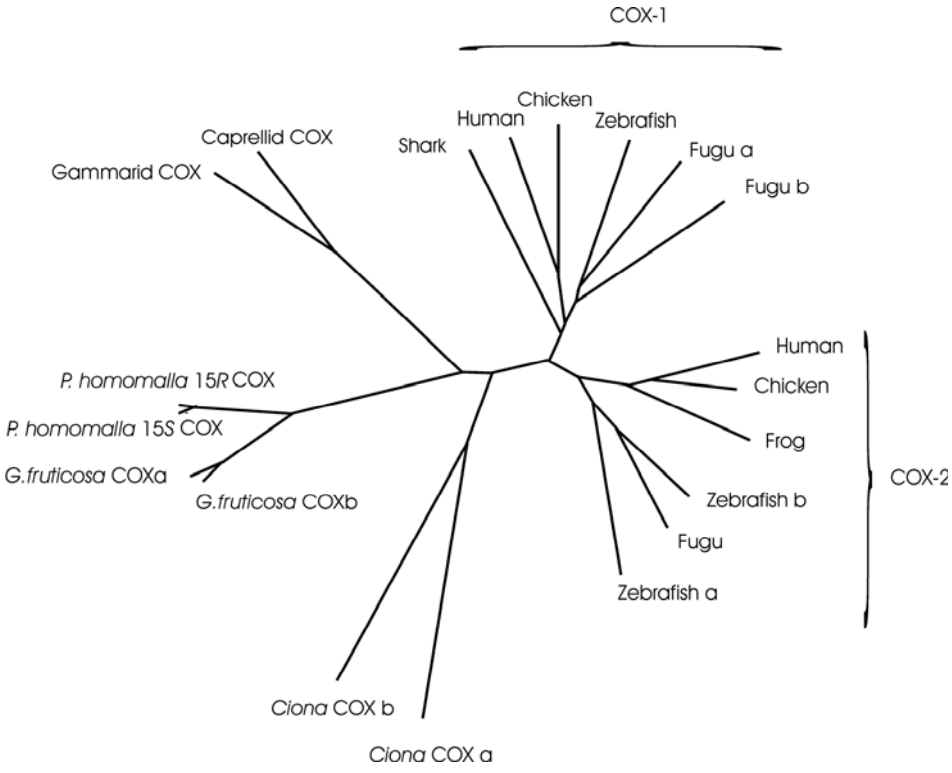


Fig. 8. Unrooted phylogenetic tree of representatives of different vertebrate and invertebrate COX sequences was constructed using CUSTALW program.

C-terminus to COX-1 sequences. Deduced protein sequences based on genome projects have been published recently.[164] According to this, a pufferfish, fugu *T. rubripes,* has three different COX sequences: one that is more similar to COX-2 and two which are similar to COX-1. The genomes of Japanese killifish, medaka (*Oryzias latipes*) and *Tetraodon nigroviridis* also contain one

COX-2 and two COX-1-like sequences and in the genome of rainbow trout there are one COX-1 and two COX-2-like sequences. [190] Most recently a second COX-2 gene ortholog has also been cloned and expressed from zebrafish.[190] An analysis of the genomes of sea squirts *Ciona intestinalis* and *Ciona savignyui* also shows the presence of two different genes. Two COX genes with 94% of the sequence identity have been cloned from the coral *G. fruticosa*. Those genes have been emerged from gene duplication independent of the vertebrate COX-1 and COX-2 divergence. The coral COXs may be common ancestors of the vertebrate COX isozymes and the divergence of the two isoforms occurred later, during the separation of vertebrates from invertebrates.[127,164] (Fig. 8) Most recently, in our laboratory two different COX were cloned from amphipodes (unpublished results).

3.2 The origin of prostaglandins in the coral *P. homomalla*

Currently two genes from two variants of *P. homomalla* were cloned, one of them coding for 15*R*-prostaglandin forming proteins and the other for 15*S*. There have been reports *of P. homomalla* colonies, where both 15*R*- and 15*S*-isomers are present.[187] Principally there are three possibilities for how this could have happened. The physical organisation of all corals comprises hundreds or thousands of individuals in a colony. If a mixture of prostaglandin epimers is found, then maybe some animals express the 15*R*-specific while the others show 15*S*-specific variant of COX. The second possibility is that there are two genes coding for proteins with opposite stereospecificity simultaneously expressed in one animal, and the third and most likely possibility is that there is yet another variant of COX protein that functions with less stringent stereocontrol for oxygenation at carbon 15. If we presume that 15*R*-COX evolved as a consequence of several mutations of 15*S*-specific variant, then maybe the COX that catalysis synthesis of mixture of prostaglandin stereoisomers has adapted only part of the mutations necessary to completely invert the stereochemistry of products.

The majority of prostaglandins in *P. homomalla* preparations are mono and bis-esters of PGA_2. Free acids of PGA_2 or free acids or esters of PGE_2 have been isolated in smaller quantities. As a result of our studies a potential mechanism of how the endogenous prostaglandins of *P. homomalla* are synthesised, was proposed (Fig. 9). The bisoxygenation of arachidonic acid via the cyclooxygenase pathway in the coral leads to prostaglandin endoperoxide and then to PGE_2 as the initial major product. Most of the PGE_2 is converted into methyl ester and then acetylated enzymatically by lipases. The acetylation drives the conversion of PGE_2 to PGA_2 and so leads to the accumulation of PGA_2 methyl ester and its carbon 15 acetate. Acetylation at carbon 11 of PGE_2 methyl ester is much faster than at carbon 15.[191] The 11-O-acetyl derivative of PGE_2 is prone to the elimination of acetic acid resulting in the formation of the

PGA_2 and it may also be subject to lipase-catalyzed elimination.[191] Further acetylation delivers the PGA_2 methyl ester 15-acetate which then accumulates

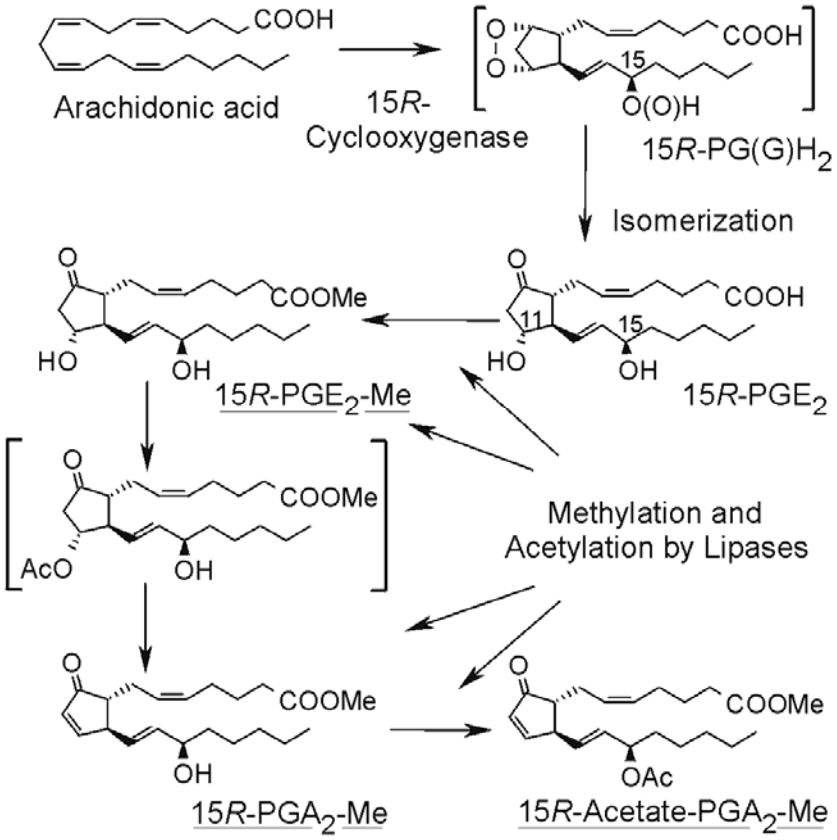


Fig. 9. Proposed mechanism for prostaglandin formation in *P. homomalla*. Prostaglandin esters identified in fresh coral extracts are underlined. Unstable intermediates are in brackets.

in the coral. This explains why the 15-acetate of PGA_2 is found in *P. homomalla*, but PGE_2 occurs only as the methyl ester. The peculiar minor isomers 5,6-trans and 13,14-cis PGA_2[192] are most likely formed by isomerisation in the sunlight of the shallow Caribbean waters.

3.3 Homology-based modelling

The tertiary structures of COX enzymes from *P. homomalla* were constructed using Swiss-Model servers and RasMol 2.6 was used for the visualisation of the tertiary structures. Ovine COX-1 numbering was used. The predicted tertiary structure of the *P. homomalla* COX enzymes was compared with the known crystal structures of mammalian cyclooxygenases COX-1 and

COX-2.[8,7] The characteristic structural units including the EGF-like module, the membrane-binding motif, and the large globular catalytic domain with

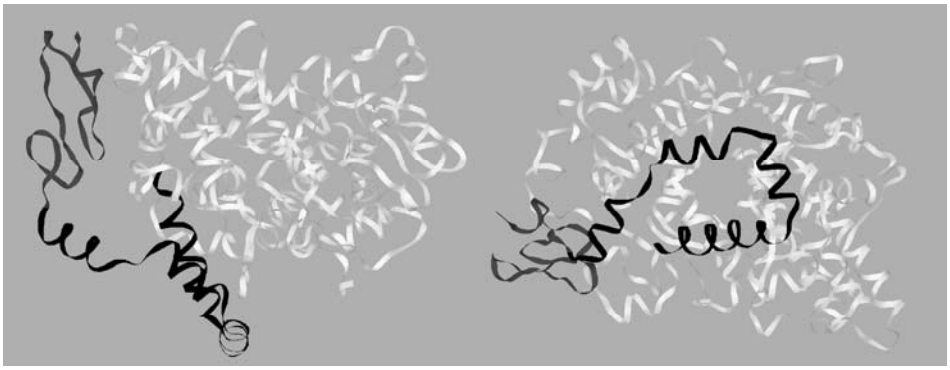


Fig 10. The predicted tertiary structure of 15*R*-COX from *P. homomalla*. The epidermal growth factor-like domain is grey, the membrane binding domain consisting of four consecutive helices is black, and the big globular catalytic domain is white. On the righthand side of the picture: a view of the enzyme molecule from the membrane side of the molecule.

cyclooxygenase and peroxidase active sites are present in the coral COX enzyme (Fig. 10). Analysis of the primary and predicted tertiary structures of *P. homomalla* COX enzymes shows strict conservation of the tertiary structure (Fig. 11), and the catalytically important amino acids in the cyclooxygenase active site (Fig. 12). The fatty acid substrate binds to the active site in an L-shaped conformation that perfectly complements the protein cavity in the

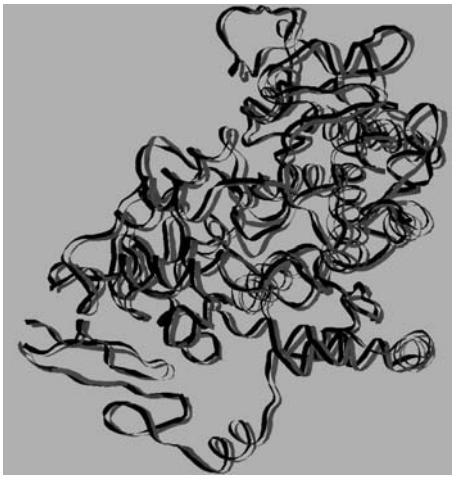


Fig 11. Superposition of the ovine COX-1 model (1DIY) (grey) and the predicted tertiary structure of *P. homomalla* 15*R*-COX (black) shows that the enzymes are superimposable.

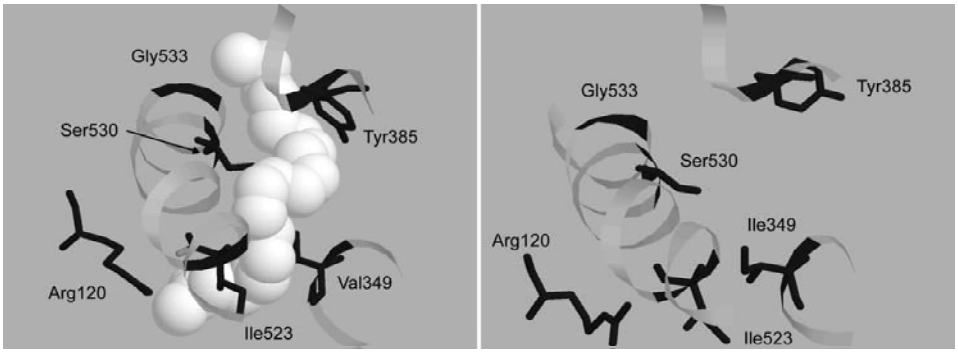


Fig 12. Cyclooxygenase active sites of ovine and coral COX enzymes. Ovine COX-1 (1DIY) on the left panel is shown with arachidonic acid (spacefill mode) bound to the active site. Some of the catalytically important amino acid residues such as catalytic Tyr385, Ser530 - the target of an aspirin attack, the carboxylate-binding residue Arg120, etc. are given in wireframe mode.

cyclooxygenase active site. The structural landmarks of the active site the catalytic Tyr385, the target of aspirin attack Ser530, the carboxylate-binding Arg120 and almost all other amino acids in the active site, such as tyrosines 355, 348, and, 504, Gly533, Ile523, *etc*, are present in the COX proteins from coral *P. homomalla*. We did not find any amino acid differences that could lead to changes in substrate positioning. In spite of the high overall homology between mammalian and coral COX enzymes, some more or less significant differences were found. Parts of the membrane-binding helices of the coral enzyme appear to lack the regular interchange of hydrophobic and hydrophilic residues suggesting possible differences in the strength of membrane association between the mammalian and coral proteins (Fig. 13). This is in accordance with the finding that it is very hard to solubilise coral COX from membrane, and during this process enzyme easily loses its activity.

Differences were found in the possible N-glycosylation sites. Only one of the conserved glycosylation sites in the COX isozymes, at Asn144, is present in the *P. homomalla* enzyme. There are two additional consensus sequences for N-glycosylation (Asn193 and Asn396), but their location differs from the consensus sequences in COX-1 and COX-2. Furthermore, the glycosylation site conserved in all known COX structures and located in the EGF-like domain (at Asn68) is missing altogether in the *P. homomalla* enzymes.

The peroxidase site of the coral COX preserves most of the catalytically vital residues like the proximal heme ligand His388 and residues His207, Gln203 from the distal side of heme that are predicted to hydrogen bond the 15-hydroperoxy group of PGG_2. At the same time there are several amino acid differences in coral enzymes that hint to the possibility that PGG_2 may not be such a good substrate to coral COX as it is to mammalian enzymes. Due to the high activity of heme, PGG_2 or PGH_2 have never been crystallised to the

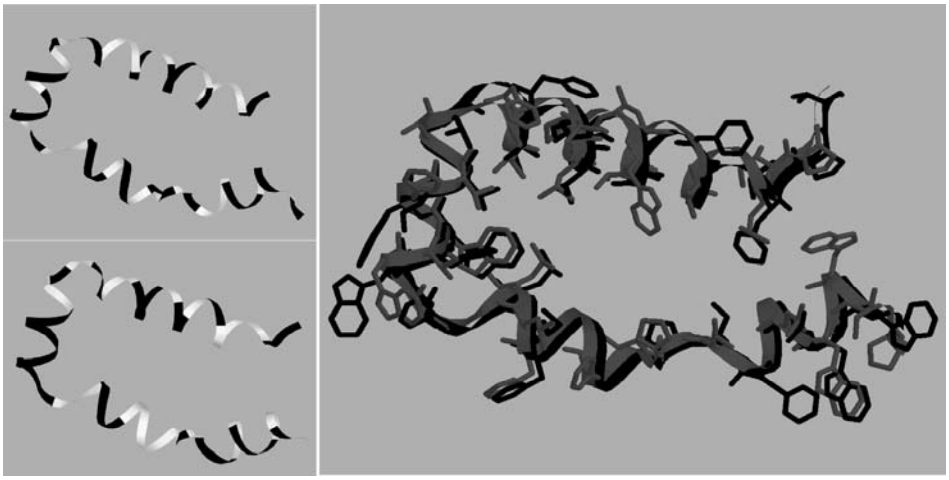


Fig. 13. The membrane binding domain structures of ovine COX-1 and *P. homomalla* COX. On the lefthand panels the interchange of hydrophobic (black) and hydrophilic (white) amino acid residues is shown separately for ovine (upper) and *P. homomalla* (lower) enzymes. On the righthand panel the MBD of ovine (grey) and coral (black) COX are superimposed. The hydrophobic side chains protruding from helices are given in wireframe mode. These amphipatic α-helices integrate into only a single lipid layer and form a monotopic membrane binding domain.

peroxidase site. Molecular docking of the substrate to the peroxidase site places PGG_2 so that Lys215 and Lys222 forming a cationic pocket, bind the carboxylate group. The salt bridges between the carboxylate oxygens and lysines are proposed to play a critical role in enzyme-substrate binding.[94] In coral COX the lysines are replaced with histidine and tryptophan. There are also differences in the shield covering the active site and in the putative PGG_2

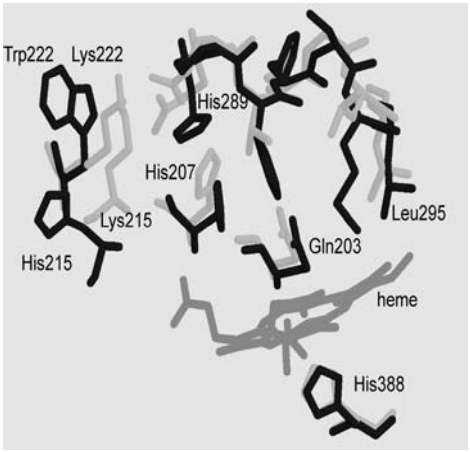


Fig. 14. Superposition of the peroxidase active site of ovine and coral COX enzymes. The proximal heme ligand His388, and the catalytically important residues His207 and Gln203 from the distal side of heme are also conserved in coral enzymes. Hydrophobic residues forming a dome over the distal side of the heme differ between coral and mammalian of COX-enzymes. The bulkier residues of *P. homomalla* (shown as darker areas here) should account for a reduction of the available space in the peroxidase site and may account for reduced peroxidase activity.

binding site. Molecular modelling of residues 289-295 shows that there are more bulky amino acids over the heme in the coral enzyme, with potential ramifications for substrate access to the peroxidase active site (Fig. 14).
The differences in the peroxidase active site are in good accordance with our previous findings that something is "wrong" with the peroxidase activity of coral COX. As a difference from mammalian COXs, a significant accumulation of PGG_2 was detected in short time incubations with arachidonic acid of native preparations of *G. fruticosa.*[185] Preparations of recombinant *P. homomalla* enzymes also revealed no peroxidase activity in standard spectrophotometric peroxidase assay.

The main difference in the cyclooxygenase active site structures of mammalian COX-1 and COX-2 is the presence in COX-2 of a bigger side pocket near the carboxylate-binding region,[8,7] due to an Ile523Val substitution that makes it accessible to larger substrates and COX-2 specific inhibitors.[193,194] The other differences between COX-1 and COX-2 in this region are in amino acids 513 and 434 (Arg513 and Val434 in COX-2 versus His513 and Ile434 in COX-1). Altogether these differences permit the selective inhibition of COX-1 and COX-2.[1] The predicted size of the hydrophobic channel of the *P. homomalla* COX enzymes is more similar to COX-1, with more bulky amino acids, Ile523 and Met434, obstructing the side pocket. This is in good accordance with the finding that the biosynthesis with *P. homomalla* enzymes was inhibited by the COX-1 selective inhibitor indomethacin, but not with the COX-2 inhibitor nimesulide. The effect of a substitution of polar amino acids at the 513 position (histidine or arginine) to neutral leucine is unclear and needs further investigation. Similarly, the conserved residue Glu524, implicated in the binding of certain inhibitors, is represented by leucine in the coral enzyme. When the size of the active site distinguishes mammalian COX-1 and COX-2 then the insertion of eighteen amino acids in the C-terminus of COX-2 is a more general rule. Most recently it was shown that this insert targets the enzyme towards rapid degradation, also being one possible way of the regulation of COX-2. The length of the C-terminus of corals COX is also closer to the constitutive vertebrate isozymes, being two amino acids longer than the C-terminus of the mammalian constitutive isoform and therefore about seventeen residues shorter than the inducible COX-2 isozyme. The C-terminal signal of *P. homomalla* is two amino acids longer than that of another coral, *G. fruticosa*. This can be one reason why in contrast to *G. fruticosa* no COX protein has ever been detected in preparations of *P. homomalla.*

3.4 Expression and product analysis(Articles I and II)

Fully sequenced coding regions of *P.homomalla* COX isozymes were expressed in the Bac-to-Bac expression system according to the manufacturer's protocol. Briefly, 15*R* and 15*S*-COX sequences were subcloned into the *Bam*HI

site of pFASTBAC1 donor plasmid and transformed into DH10Bac cells. Recombinant bacmid was isolated and transfected into Sf9 insect cells, and the resulting recombinant baculovirus was used for protein production. Cells with a density of 1.2-1.8 × 10^6 cells/ml were infected with a multiplicity of infection (moi) of about 0.02. When a higher virus titer was then used, despite the increase in expressed protein levels, the catalytic activity of the enzyme decreased dramatically. (According to the available literature, the virus titers of up to twenty moi were used for the production of mammalian COX-2.[195]) This is probably because of the limited ability of insect cells to properly glycosylate the protein. Up to 72 hours post-infection the insect cells were found to be healthy and viable. Then they were harvested and analysed with Western blotting for the presence of protein (the coral COX enzymes were found to cross-react with a commercial monoclonal antibody which had been raised against murine COX-2). Western blot (Fig. 3 in Article I and Fig 1 in Article III) showed three closely migrating bands, most probably reflecting different glycosylation levels.

Kinetic measurements were conducted with a fibre-optic oxygen sensor (Article III). The K_m values were determined for arachidonic acid with the microsomal fractions of both wild type enzymes. The values were quite similar. 15*S*-COX had a K_m value of 5.8±1.7 μM, which is the same or slightly smaller than the K_m of a similar preparation of ovine COX-1. The K_m value for 15*R*-COX was somewhat higher, at 8.7 ±2.4 μM. Hence, despite the differences in product stereochemistry, the kinetic properties of the isozymes are similar.

The catalytic activities of recombinant enzymes were compared by incubations with 50 μM radiolabelled arachidonic acid using equal amounts of either Sf9 cell pellet or a microsomal fraction of cell preparation.

In optimum conditions a suspended cell pellet of one million cells was able to synthesise approximately 7-10 nmole prostaglandins. This was taken as the maximum activity level when different wild-type or mutant recombinants were compared.

In most experiments, designed to simplify the product pattern, 0.5 mM $SnCl_2$ as an aqueous suspension was added to the incubation mixture just before the substrate. This gave an immediate reduction of the prostaglandin endoperoxides, the primary products of COX enzymes, to corresponding hydroxy compounds.

The products were analysed and quantified by TLC and/ or HPLC analysis. The TLC analysis was conducted using the solvent system of benzene/dioxane/acetic acid for separation and an anisaldehyde reagent for visualisation of the spots. The 15*S* and 15*R*-epimers of prostaglandins were easily distinguishable by their R_f values and characteristic colours (Fig 2 and Table 1 in Article II). RP-HPLC analysis of the products of recombinant 15*S* and 15*R*-COX were conducted using a solvent of water/acetonitrile/acetic acid (Fig 15).

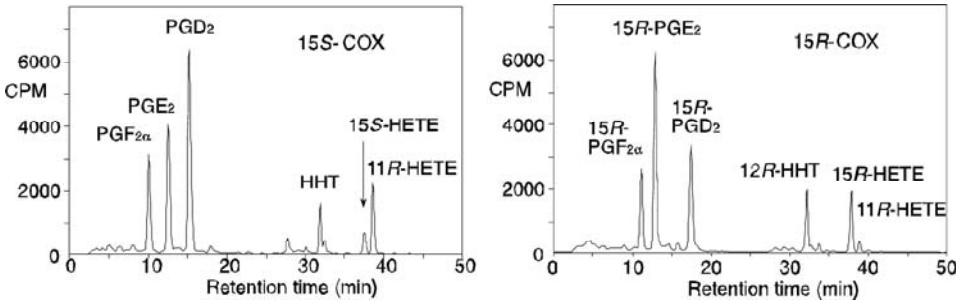


Fig. 15. RP-HPLC analysis of [1-^{14}C]arachidonic acid metabolites of 15*S* and 15*R*-coral COX expressed in Sf9 insect cells. The incubation products were run on a Waters Symmetry C_{18} column (25 × 0.46 cm) using a solvent of water/acetonitrile/acetic acid (62.5/37.5/0.01, v/v/v, changed to the proportions 30/70/0.01 at 25 min) at a flow rate of 1 ml/min and using a Radiomatic Flo-One detector for on-line recording of radioactivity.

This afforded a good resolution of prostaglandins from different groups (PGE_2, PGD_2, and $PGF_{2\alpha}$) and monohydroxy acids as well, but not 15*R/S*-stereoisomers of $PGF_{2\alpha}$. The epimers were also inseparable when methanol was used as an organic modifier. In reversed phase chromatography the use of two modifiers (ternary eluents) frequently improves selectivity. The solvent system that afforded separation of carbon 15 stereoisomers is a ternary eluent of water, methanol and acetonitrile, at a constant proportion of methanol and acetonitrile (equimolecular mixture) containing up to 50% by volume of 0.01 M H_3PO_4 (Fig. 16)

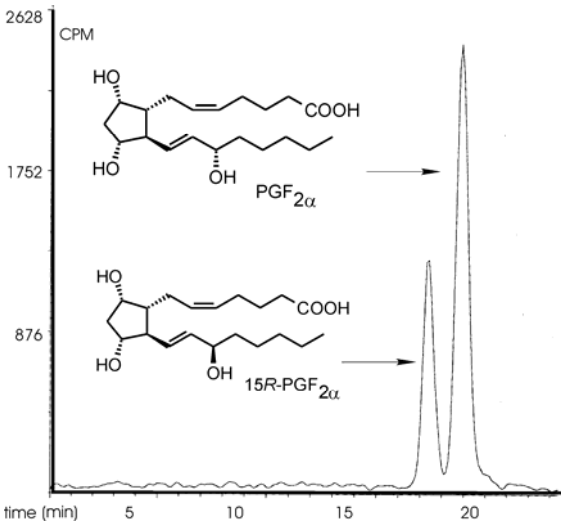


Fig. 16. The separation of stereoisomeres of $PGF_{2\alpha}$ by RP-HPLC.
The mutant enzymes were expressed in insect cells and analysed for product stereochemistry. The separation of products of the mutant M2 are shown. Waters Symmetry C_{18} column (25 X 0.46) with the solvent system of MeOH/AcN/0.01 M H_3PO_4 (22 : 28 : 50, v/v/v), and a Radiomatic Flo-One detector were used.

The chromatographic analysis showed that the recombinant enzymes cloned from two varieties of *P.homomalla* formed prostaglandins with the opposite configuration of carbon 15. The COX enzyme cloned from *P. homomalla* samples collected in the Bahamas synthesises prostaglandins with the regular 15*S* configuration, whereas the enzyme cloned from the same species collected in the Florida Keys forms prostaglandins with the unique 15*R* configuration. The stereochemistry matched the endogenous prostaglandin contents extracted from the fresh coral samples.

The identity of the reduced product of 15*R*-COX with 15*R*-$PGF_{2\alpha}$ was established by high-field (11.7 T) NMR spectroscopy (Article I).

The results established that two slightly different COX-genes are present in two *P. homomalla* varieties of different geographical locations, and that enzymes coded by these genes are responsible for the biosynthesis of prostaglandin stereoisomers in these coral.

3.5 The stereospecificity of oxygenation reaction. Mutagenesis studies (Articles II and III)

Most studies of COX enzymes using site-directed mutagenesis have been carried out in order to identify residues involved in substrate or inhibitor binding, or initiating catalysis. However, besides these issues an important question in prostaglandin formation is how does the enzyme control the regiochemistry and stereochemistry of fatty acid oxygenation. The cyclooxygenase reaction not only differs from the non-enzymatic oxidation of arachidonic acid in the increased reaction rate but also in the high degree of stereochemical specificity, as one of the 32 possible isomers predominates.[23] During this bisoxygenation, an achiral polyunsaturated fatty acid is oxygenated twice, one carbon-carbon and three carbon-oxygen bonds are formed, and five chiral centres are introduced.[1] Currently there is far more understanding about the stereochemistry of the first oxygen attack than about the second, which is the one that introduces the hydroperoxy group to the carbon 15. Prostaglandins with a 15*S*-configuration are molecules utilised in signalling by vertebrates from fish to mammals. The correct stereochemistry of the signal molecule is crucial for its biological activity and so is under a strict enzymatic control.[196] Another aspect in prostaglandin metabolism is the need to utilise the signal molecules. The biologically active prostaglandis are further metabolised into decomposition products that are subsequently eliminated from the body by excretion in urine.[197] As with the biosynthesis of prostaglandins, their metabolism and degradation has to occur rapidly. The initial step in catabolism of prostaglandins is oxidation of the 15-hydroxy group, which is catalyzed by 15-hydroxyprostaglandin dehydrogenase (PGDH). It has been shown that this enzyme is also stereospecific with regard to configuration at carbon 15. PGE_1 with 15*R*-hydroxy group was shown not to be a substrate, but an inhibitor of PGDH.[198] Furthermore, 15-HETE, the only compound formed upon the acetylation of COX-2 (aspirin inhibition) occurs exclusively with the *R*-

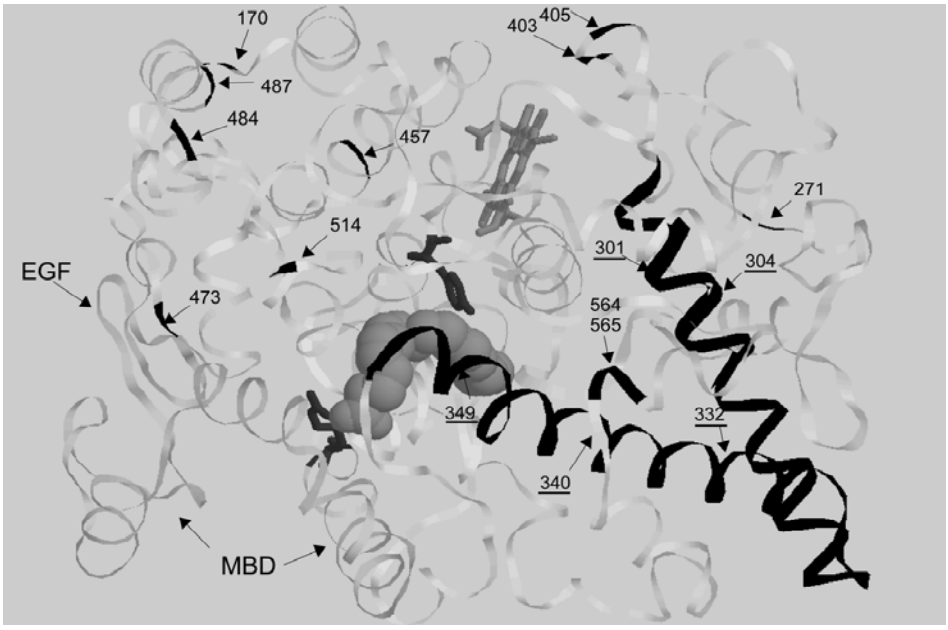


Fig. 17. The location of amino acid differences are shown on the ovine COX-1 model (1DIY) where catalytically competent arachidonic acid (spacefill mode) bound in a cyclooxygenase active site and heme (wireframe mode) in peroxidase site are shown. The membrane-binding domain (MBD) and epidermal growth factor-like (EGF) domain are darker grey. Amino acid residues Arg120, and Tyr385, important for substrate binding and catalysis respectively are in wireframe mode. Two successive helices 5 and 6, which constitute the main body of cyclooxygenases, are shown in black. Amino acid differences in those helices are underlined.

configuration. Therefore the mechanism of how COX controls the stereochemistry of the carbon 15 is of ultimate importance. We were interested, therefore, in determinants to the stereospecificity of the second oxygen attack and whether this is reversible by mutagenesis.

The identification of an enzyme that catalysis the transformation of arachidonic acid to 15*R* prostaglandins should provide insights into the mechanism of stereocontrol in the oxygenation reaction. Furthermore, the fact that two enzymes that synthesise prostaglandins with an opposite stereochemistry at carbon 15 differ only in seventeen amino acids gives us an excellent chance to figure out the residues responsible for the stereocontrol. With this aim in mind, a series of sequence swapping and site-directed mutagenesis experiments between 15*R*- and 15*S*-COX were performed.

The site-directed mutations were performed by PCR using the overlap extensive method,[199] (Mutants M1, M2, and M9-M20). The PCR-generated fragments were inserted into the pFASTBAC1 vector in the *Bam*HI restriction site. Extra restriction sites were introduced for the selection of mutated clones (*Hpa*I, *Stu*I, *Hind*III, and *Vsp*I).

Parts of cDNA were swapped by using common restriction sites. Restrictases *Bam*HI, *Nco*I, *Xba*I and *Ban*I were used. The mutations were performed within the pFASTBAC1 vector, or the mutants were subcloned in pGEM-T-Easy vector (mutants M3-M8). The success of swapping was checked by restriction analysis using restrictases that selectively cut only one isoenzyme (*Bcl*I and *Hinc*II).

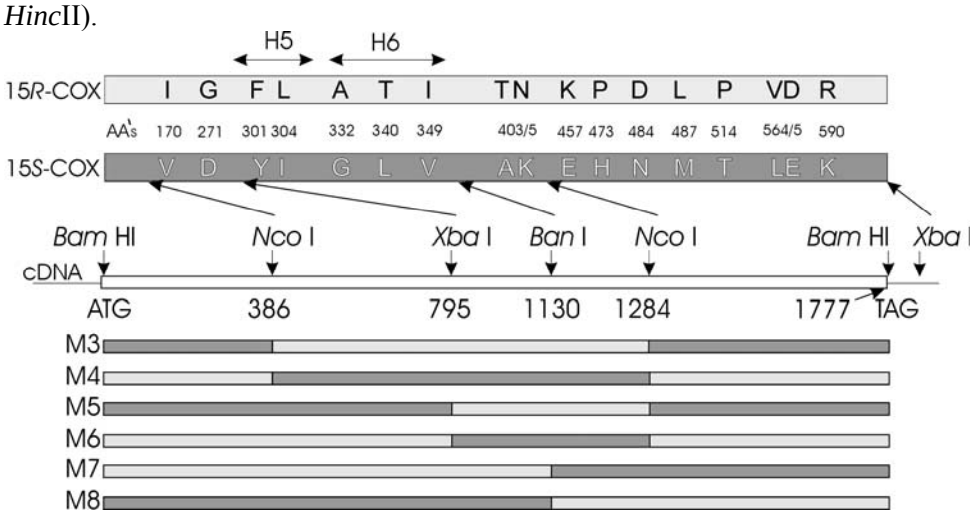


Fig. 18. **A schematic drawing of amino acid differences between 15S and 15*R*-COX enzymes**. Residues characteristic to 15*R*-COX are given in black on light background and 15*S*-COX residues are in inversed mode. **Partial restriction map and sequence swapping strategy for 15*S* and 15*R*-COX genes from *Plexaura homomalla***. *P. homomalla* COX genes were digested with restriction enzymes shown in the illustration and the sequences were swapped between counterparts. The resulting mutants M3-M8 are given as schematic drawings where lighter areas depict the parts of sequences which are derived from the 15*R*-COX and the darker areas are those of the 15*S*-COX.

The resulting mutant/chimeric proteins were expressed in the baculovirus/ insect cell expression system, and subsequently the cell homogenates were incubated with substrate and the products were analysed.
A comparison of primary structures shows that the differences between 15*S* and 15*R*-specific *P. homomalla* COX proteins are dispersed along the polypeptide chain with most of them located in the C-terminal half of the protein (Fig. 7).
Among the total seventeen amino acid differences there is only one residue, Val349, which is conserved throughout all known COX proteins with 15*S*-stereospecificity. Val349 is replaced with isoleucine in the 15*R*-COX. This residue lies in the arachidonate-binding channel of the COX proteins and is proposed to be critical for the positioning of the substrate in a conformation that yields PGG_2. X-ray analysis of COX-2 crystallised together with the prostaglandin H_2 bound to the cyclooxygenase active site (1DDX;[64]), revealed a 3.087 Å distance between the Val349 and the 15-hydroxyl group of the PGH_2 so making this residue the main candidate of oxygenation stereocontrol at

carbon 15. Ile349 in 15*R*-COX was replaced with valine, the natural resident of a regular 15*S*-specific COX. The mutant enzyme (M1) expressed in baculovirus system converted arachidonic acid to a mixture of 15*R* and 15*S*-prostaglandins. TLC and HPLC analysis revealed that this single mutation inverted the product stereochemistry from nearly 100% of *R*-isomer to 70% of *S*-isomer.

Converse mutation to 15*S*-making COX (V349I) gave an enzyme (M2) synthesising 65% of *S*- and 35% of *R*-prostaglandins. We emphasise that the effect of the mutation was not as big as in the case of the I349V mutation of *R*-making *P. homomalla,* and matches with the earlier findings that changing valine-349 in human COX-1 to isoleucine delivered an enzyme that formed ~40% of 15*R*-prostaglandins.[104]

As the mutation of the active site Val349/Ile349 did not completely reverse the product stereochemistry, we started to search for additional stereospecificity determinants from the seventeen amino acid differences. The other sixteen residues are dispersed all over the entire protein, far from the active site, mostly on the surface of the catalytic domain (Fig. 17). To limit parts of the primary structure which may influence the carbon 15 chirality, we prepared several chimeras between *S*- and *R*-variety *P. homomalla* cyclooxygenase sequences (Fig. 18). High sequence similarity enabled the use of common restriction sites to swap parts of sequences between enzymes.

The first restriction enzyme used was *Nco*I, which cuts out the piece of COX cDNA, corresponding to amino acid residues from Tyr138 to His440. These sequence regions containing nine of a total of seventeen differences were swapped between 15*R* and 15*S*-specific COX sequences. 15*R*-specific COX with nine changed amino acid residues (mutant M4) started to make almost only 15*S* prostaglandins (>95%, Table 2). Analogous 15*S*-COX with an insertion of nine amino acid residues from 15*R*-specific COX (mutant M3) formed 85% of 15*R* prostaglandins.

The nine suspects were narrowed down to seven by using *Xba*I restrictase. As the DNA inserts were cloned to the *Bam*HI site in the pFASTBAC1 vector, the restriction with *Xba*I enables the swapping of sections of DNA between different clones. *Xba*I cuts between the nucleotide corresponding to the amino acid residue Tyr275 and the multiple cloning site in the vector. The wild type clones and mutant constructs M3 and M4 were subjected to restriction with *Xba*I, the sequences were swapped and chimeras were obtained where fragments from the mutant constructs M3 and M4 were exchanged into wild-type clones so that seven out of seventeen amino acid residues (Phe/Tyr301, Leu/Ile304, Ala/Gly332, Thr/Leu340, Ile/Val349, Thr/Ala403, and Asn/Lys405) were swapped between counterparts. The products of the second series mutant enzymes M5 and M6 produced approximately the same composition of products (20 and 95% of *S*-isomer respectively) as previous ones (M3 and M4), meaning that amino acid residues 170 and 271 have no significant influence on oxygenation stereospecificity. In a similar way the influence of amino acid residues 403 and 405 was excluded. Wild-type cDNA sequences were restricted with *Ban*I restrictase and the parts of sequences were

swapped. In this case subcloning had to be used as *Ban* I cuts three times within the vector pFASTBAC1. As a result chimeric enzymes where the C-terminal regions beginning from amino acid Trp387 were interchanged between isoenzymes were obtained (mutants M7 and M8). The chimera with the N-terminal part of 15*S*-COX and the C-terminal part of 15*R*-COX (M8) gave a product profile similar to the regular 15*S*-specific COX. In the opposite case, the chimera with *R*-specific N-termini, and *S*-specific C-termini (M7) formed prostaglandins with 85% of *R*- and 15% of *S*-configuration. Comparison of the product composition with those from the aforementioned mutants (M3 and M4) precluded the influence of amino acid residues 403 and 405.

Altogether, the results of the sequence-swapping experiments indicate that the *S*-configuration of carbon 15 of prostaglandins is determined by the section of protein where five out of seventeen amino acid differences between 15*S*-COX and 15*R*-COX reside. Interestingly, the opposite case when replacements were made in *S*-specific cyclooxygenase the mutants continued forming a small percentage of 15*S*-prostaglandins (≈15%). It also became apparent that mutants which were derived from 15*R*-COX were less active and tended to lose activity more easily. Therefore, the 15*S*-configuration of prostaglandins seems to be evolutionary preferred. And if we can speculate that 15*R*-COX evolved as a consequence of a series of mutations in 15*S*-COX, then this explains why there are so few descriptions of colonies containing mixtures of stereoisomers. It probably needs several co-mutations to obtain a functional and stable enzyme with changed stereospecificity.

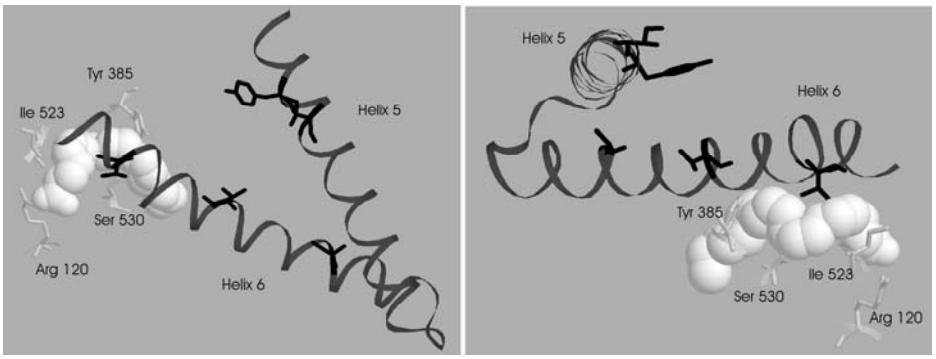


Fig. 19. Part of ovine COX-1 (1DIY) with substrate arachidonic acid (spacefill mode) bound in the cyclooxygenase active site. Catalytically important amino acids are shown in the wireframe mode. Helices 5 and 6 are shown with amino acid differences between 15*R* and 15*S*-COX highlighted in wireframe mode.

Homology modelling of the enzyme structures (Fig. 19), shows the protein region where those decisive differences are located consisting of two consecutive helices, 5 and 6 (the nomenclature for the helices is based on the spatial equivalence of the secondary structure between myeloperoxidases[7]). Those two long helices forming the helix-turn-helix motif,[72] traverse through

the entire protein and form a frame to the whole catalytic domain. The alignment of the primary sequences of cyclooxygenases from different organisms demonstrates that those helices are the most highly conserved regions in COX proteins (Fig. 20). The amino acid sequence identity of the whole enzyme between COX-1 and COX-2 is 60-65% within the same species while the identity among orthologs from different vertebrate species varies from 85-90%.[55] Conservation is much higher within the helices 5 and 6 being 85-90% for isozymes and 95-100% for orthologs. A total of 50% of amino acids residing on those helices are absolutely conserved between all known cyclooxygenases. A nine-residue-conserved peptide in the middle of helix 5 is also present in myeloperoxidase.[88] The structural or functional significance of such a high residue conservation has not been elucidated yet, but it has been shown that mutations on those helices can have dramatic effects on both cyclooxygenase and peroxidase activities.

For instance, the mutation of cysteine at position 313 to serine (changing a single sulfur atom to oxygen) reduces the cyclooxygenase and peroxidase activities of COX-1 by 90%,[86,85] and H309Q or H309A mutations resulted in a complete loss of both the peroxidase and cyclooxygenase activity of COX-1.[96] Unlike in other cyclooxygenases, in *P. homomalla* the aforementioned region (helix-turn-helix region of helices 5 and 6 Fig. 21) is the least conserved part of the entire protein between 15*S* and 15*R*-cyclooxygenases. Five of seventeen differences between isoenzymes are situated on those two helices which means that compared to the 97% overall identity, the conservation within helices 5 and 6 is only at 90%. This is probably not accidental, but reflects the importance of helices 5 and 6 in maintaining the desired configuration of carbon 15 in two variants of *P. homomalla* COX proteins.

To figure out which amino acids among those five (besides Val/Ile349) play a role in stereocontrol, a series of site-directed mutations were made with amino acid residues exchanged between counterparts. The first mutations were carried out with amino acids residing on the same helix with Val/Ile 349 (helix 6). Besides residue 349 there are two more residues by which the helix 6 of 15*R* and 15*S*- specific COX differ. They are glycine versus alanine at position 332 and leucine versus threonine at 340.

A comparison of dozens of COX sequences from coral to mammals showed that Thr340 is a strongly conserved residue. The only exception besides the *S*-variety *P. homomalla* is another coral, *G. fruticosa*, which also has leucine at position 340. A mutation of this residue resulted in the complete loss of activity when threonine in I349V mutant of 15*R*-COX (M1) was replaced with leucine, the natural resident in 15*S*-COX (double mutant M13).

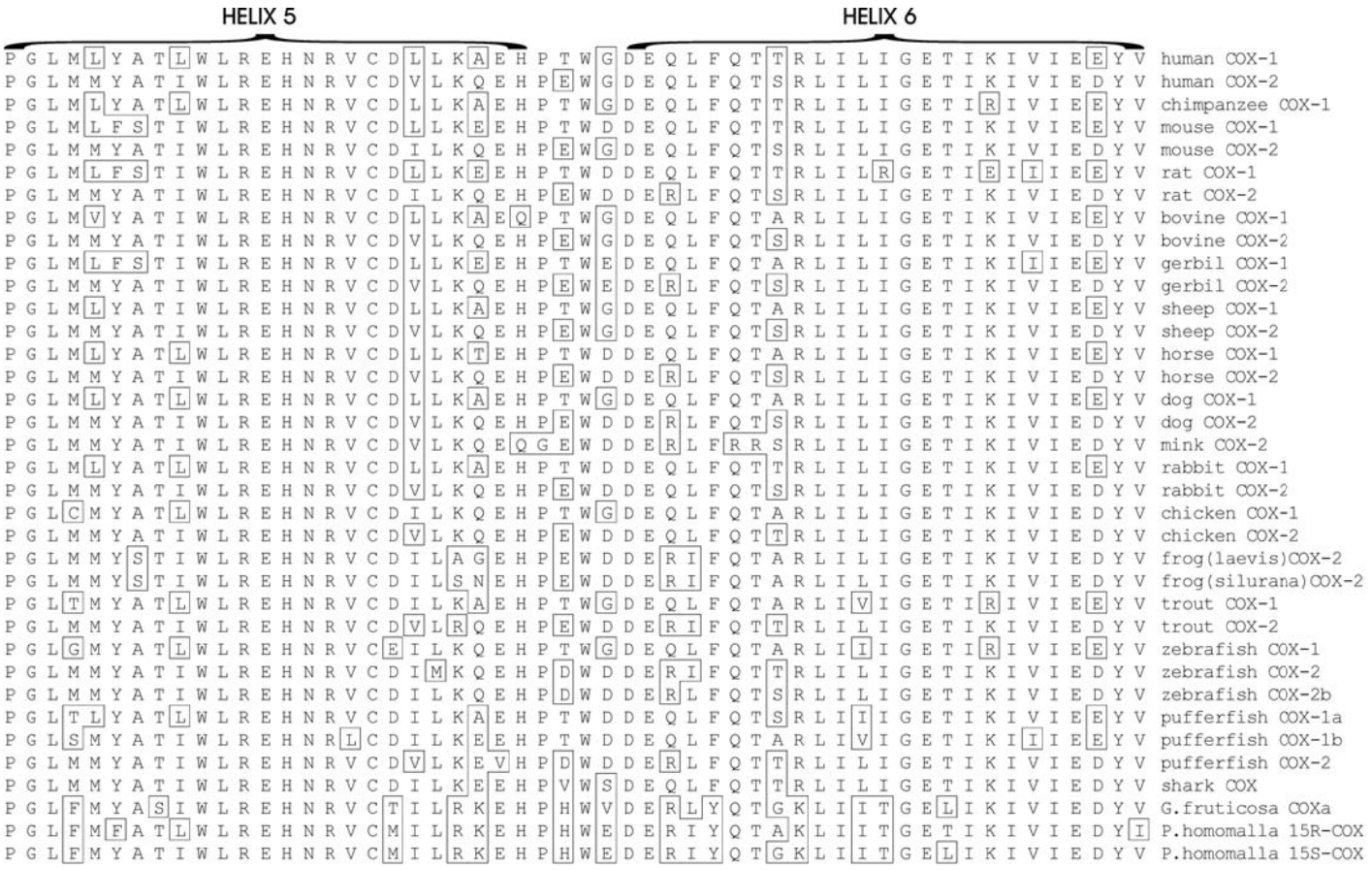


Fig. 20 A multiple sequence alignment of helices H and 6, the most conserved region of the entire protein with differences boxed.

According to the BLOSUM62 statistical and likelihood method,[200] in analysing amino acid substitutions, leucine to threonine belongs to a very unlikely substitution. It may need a compensatory mutation somewhere else but this has yet to be located. Attempts to use statistical methods to find co-mutated sequences were hampered by the rather limited number of COX sequences currently available. We can only suspect that the partner could be Tyr/Phe at position 301, as all mutants comprising phenylalanine at position 301 and leucine at position 340 were inactive (M12, M13, M19). It seems that at least one of these two residues needs to have a hydroxy group in the side chain.
Furthermore, according to the multiple sequence alignment of all known COX proteins, the presence of phenylalanine (instead of tyrosine) in position 301 (mouse, rat, gerbil) is always accompanied by serine in position 302 (instead of alanine); so it seems likely that the conservation of polarity (hydroxy group) at the beginning of helix 5 has some functional or structural significance. The converse mutation in V349I 15*S*-COX (M2) in which leucine 340 was mutated to threonine (M16) did not affect the activity or stereospecificity of the reaction.

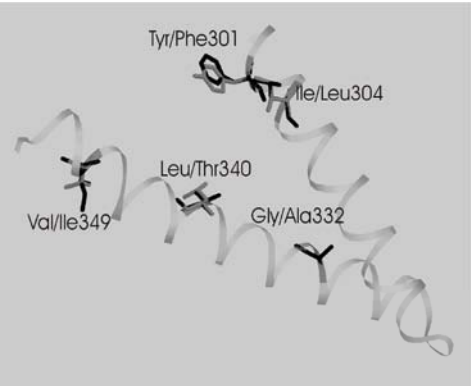


Fig. 21. Superposition of helices 5 and 6 of *P. homomalla.* Amino acid differences between isozymes that are the main determinants of stereospecificity are given in fireframe mode.

The MSA of cyclooxygenases demonstrates that position 332 is not very strongly conserved. (Fig. 20) Smaller amino acid residues like glycine, alanine, serine, and threonine are all tolerated. Curiously, when the G332A mutation was subjected to the V349I mutant of *R*-specific COX (M1), the resulting double mutant M14 was inactive. The converse double mutant A332G/I349V of 15*S*-specific COX (M15) was at least partly active but had product stereospecificity similar to that of the initial single mutant I349V (M1). Single mutants of position 332; G332A of *S*-specific, and A332G of *R*-specific cyclooxygenase (mutants M17 and M18) were also prepared. Surprisingly, both mutants retained full activity but had no change in the stereospecificity of oxygenation.

An attempt to restore the activity of the M13 and M15 mutants of 15*R*-COX by changing the whole set of residues on the helix 6 differing between *S* and *R* variant COX was unsuccessful. The triple mutant of *R*-specific cyclooxygenase (M19, chimeric enzyme where the helix 6 is replaced with that of 15*S*-variety COX) was inactive (probably thanks to the aforementioned unfavourable threonine-leucine interchange at position 340). The converse mutant (M20, 15*S*-variety COX with helix 6 of *R*-variety COX) was fully active with the stereochemistry of the products nearly the same as that of single mutant M2. Equipped with those results we concluded that the additional stereospecificity determinants have to reside on helix 5.

On helix 5 the differences between *S* and *R* variety cyclooxygenases are in two amino acids Phe/Tyr 301 and Leu/Ile 304. When these residues were mutated pairwise, which means that besides Val/Ile 349 the whole helix 5 was changed, the resulting mutants M11 and M12 were inactive. Mutating position 301 alone gave one inactive (M10) and one active (M9) mutant. The composition of products of the double mutant M9 (F301Y/I349V of 15*R*-COX) did not significantly differ from that of the single mutant I349V (M1).

Our results clearly indicate that the amino acid determinants of oxygenation stereospecificity lie on the pair of helices 5 and 6 and that helix 5 which lies quite far from the active site contributes substantially to the stereospecificity of the oxygenation reaction. Due to the tendency towards the inactivating mutations within those helices we could not specify the exact role of each single amino acid residue. It looks as though they act in a concerted mode and the helix 5, despite its distance from the active site, has an important role in the control of prostaglandin stereochemistry at carbon 15.

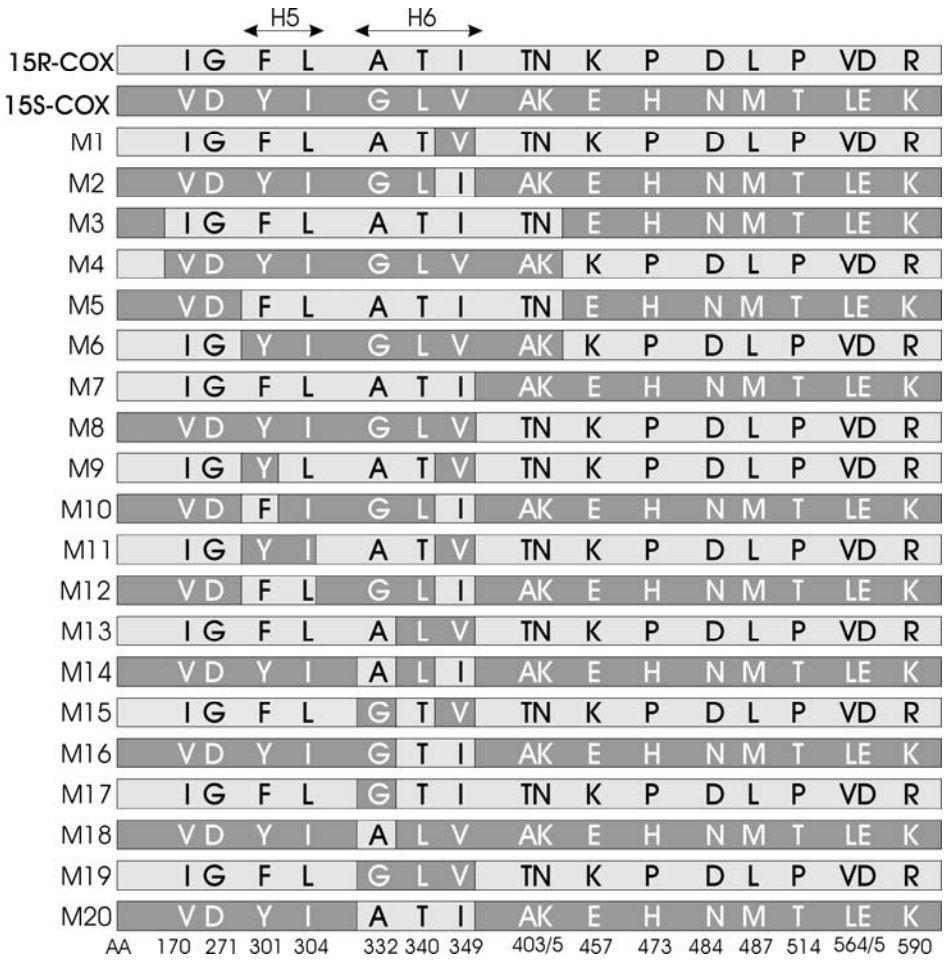


Table 2. The mutagenesis strategy, relative activity of mutants, and the composition of oxygenation products. Seventeen amino acid differences between 15*S* and 15*R*-COX which were interchanged during the mutagenesis studies are shown. Residues characteristic to 15*R*-COX are given in black on a light background and 15*S*-COX residues are in inversed mode. The residue numbering is given below and the position of helices 5 and 6 above the sequences. Swt and Rwt as templates for mutagenesis depict 15*S* and 15*R*-COX respectively.

	template	mutation/restriction	construct	%*S*	%*R*	activity
Rwt				<5	>95	+++
Swt				>95	<5	+++
M1	Rwt	I349V	Rwt;I349V	70	30	+++
M2	Swt	V349I	Swt; V349I	65	35	+++
M3	Swt+Rwt	*Nco*I		15	85	+++
M4	Swt+Rwt	*Nco*I		>95	<5	+++
M5	M3+Swt	*Xba*I		20	80	++
M6	M4+Rwt	*Xba*I		>95	<5	+++
M7	Rwt+Swt	*Ban*I		15	85	++
M8	Rwt+Swt	*Ban*I		>95	<5	+++
M9	M1	F301Y	Rwt; I349V/F301Y	75	25	+++
M10	M2	Y301F	Swt; V349I/Y301F	-	-	-
M11	M1	F301Y; L304I	Rwt; I349V/F301Y/L304I	-	-	-
M12	M2	Y301F; I304L	Swt; V349I/Y301F/I304L	-	-	-
M13	M1	T340L	Rwt; I349V/T340L	-	-	-
M14	M2	G332A	Swt; V349I/G332A	-	-	-
M15	M1	A332G	Rwt; I349V/A332G	77	23	+
M16	M2	L340T	Swt; V349I/L340T	68	32	+++
M17	Rwt	A332G	Rwt; A332G	<5	>95	+++
M18	Swt	G332A	Swt; G332A	>95	<5	+++
M19	M13	A332G	Rwt; I349V/T340L/A332G	-	-	
M20	M16	G332A	Swt; V349I/L340T/G332A	58	42	+++

Table 2 continued

CONCLUSIONS

In this thesis the biosynthetic pathways to epimeric prostaglandins in Caribbean gorgonian *Plexaura homomalla* have been described and the enzymes involved in catalysis have been cloned, functionally expressed and characterised. The principal findings of the present study can be summarised as follows:

- A 30 year-old mystery of the biosynthetic origin of 15*R*-prostaglandin esters in *Plexaura homomalla* was solved. A unique 15*R*-specific COX along with its 15*S*-counterpart were cloned and characterised from this coral. It was found that the epimeric prostaglandins identified in *P. homomalla* samples from different geographical locations are synthesised by two COX isoforms sharing a 97% amino acid sequence identity. Hence, the very minor sequence divergence accounts for the contents of epimeric prostaglandins in two variants of the coral and these differences do not arise from the isomerisation of the products.
- Using 15*R*- and 15*S*-specific COX isoforms as models for site-directed mutagenesis and sequence swapping studies, the structural determinants of stereocontrol at the carbon-15 of prostaglandins were determined. It was found that a single active site amino acid interchange (Ile349Val or Val349Ile) between two isozymes results in a partial change of prostaglandin stereochemistry at C-15.
- For the complete inversion in the oxygenation stereospecificity, four more amino acid substitutions are needed. These are so-called second shell residues which are located on two neighbouring helices, 5 and 6, far from the substrate binding site. It is concluded that this helix-turn-helix motif, which was thought to be the central building block of the peroxidase active site for proteins of the myeloperoxidase family, is involved in the control of oxygenation with 15*R* or 15*S* stereospecificity in the dioxygenases active site of cyclooxygenases.

REFERENCES

1. Rouzer, C.A. and Marnett, L.J. (2003) Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. *Chem. Rev.*, **103,** 2239-2304.

2. Ohki, S., Ogino, N., Yamamoto, S., and Hayaishi, O. (1979) Prostaglandin hydroperoxidase, an integral part of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.*, **254,** 829-836.

3. Smith, W.L. (1989) The eicosanoids and their biochemical mechanisms of action. *Biochem. J.*, **259,** 315-324.

4. Funk, C.D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*, **294,** 1871-1875.

5. Kraemer, S.A., Meade, E.A., and DeWitt, D.L. (1992) Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5'-flanking regulatory sequences. *Arch. Biochem. Biophys.*, **293,** 391-400.

6. Fletcher, B.S., Kujubu, D.A., Perrin, D.M., and Herschman, H.R. (1992) Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *J. Biol. Chem.*, **267,** 4338-4344.

7. Picot, D., Loll, P.J., and Garavito, M. (1994) The X-ray crystal structure of the membrane protein prostaglandin H_2 synthase-1. *Nature,* **367**, 243-249.

8. Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M.F. (1996) Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nat. Struct. Biol.,* **3,** 927-933.

9. Kurumbail, R.G., Stevens, A.M., Gierse, J.K., McDonald, J.J., Stegeman, R.A., Pak, J.Y., Gildehaus, D., Miyashiro, J.M., Penning, T.D., Seibert, K., Isakson, P.C., and Stallings, W.C. (1996) Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature*, **384,** 644-648.

10. Van Der Donk, W.A., Tsai, A.L., and Kulmacz, R.J. (2002) The Cyclooxygenase reaction mechanism. *Biochemistry*, **41,** 15451-15458.

11. Herschman, H.R. (1996) Prostaglandin synthase 2. *Biochim. Biophys. Acta*, **1299,** 125-140.

12. Smith, W.L., DeWitt, D.L., and Garavito, R.M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.,* **69,** 145-182.

13. Marnett, L.J. and DuBois, R.N. (2002) COX-2: a target for colon cancer prevention. *Annu. Rev. Pharmacol. Toxicol.,* **42,** 55-80.

14. Vane, J.R. and Botting, R.M. (1998) Mechanism of action of antiinflammatory drugs. *Int. J. Tissue React.*, **20,** 3-15.

15. Thomas, T., Nadackal, T.G., and Thomas, K. (2001) Aspirin and non-steroidal anti-inflammatory drugs inhibit amyloid-beta aggregation. *Neuroreport*, **12 ,** 3263-3267.

16. Murakami, M. and Kudo, I. (2002) Phospholipase A_2. *J. Biochem.* (Tokyo), **131,** 285-292.

17. Hamberg, M. and Samuelsson, B. (1967) Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. *J. Biol. Chem.,* **242,** 5344-5354.

18. Hamberg, M. and Samuelsson, B. (1967) On the mechanism of the biosynthesis of prostaglandins E_1 and $F_{1\alpha}$ *J. Biol. Chem.*, **242,** 5336-5343.

19. Kulmacz, R.J. and Lands, W.E. (1983) Requirements for hydroperoxide by the cyclooxygenase and peroxidase activities of prostaglandin H synthase. *Prostaglandins*, **25,** 531-540.

20. Shimokawa, T., Kulmacz, R.J., DeWitt, D.L., and Smith, W.L. (1990) Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J. Biol. Chem.*, **265,** 20073-20076.

21. Goodwin, D.C., Gunther, M.R., Hsi, L.C., Crews, B.C., Eling, T.E., Mason, R.P., and Marnett, L.J. (1998) Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. Detection of the radical derivative of tyrosine 385. *J. Biol. Chem.*, **273,** 8903-8909.

22. Tsai, A., Hsi, L.C., Kulmacz, R.J., Palmer, G., and Smith, W.L. (1994) Characterization of the tyrosyl radicals in ovine prostaglandin H synthase-1 by isotope replacement and site-directed mutagenesis. *J. Biol. Chem.*, **269,** 5085-5091.

23. Marnett, L.J., Rowlinson, S.W., Goodwin, D.C., Kalgutkar, A.S., and Lanzo, C.A. (1999) Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J. Biol. Chem.*, **274,** 22903-22906.

24. Karthein, R., Dietz, R., Nastainczyk, W., and Ruf, H.H. (1988) Higher oxidation states of prostaglandin H synthase. EPR study of a transient tyrosyl radical in the enzyme during the peroxidase reaction. *Eur. J. Biochem.,* **171,** 313-320.

25. Smith, W.L. and Song, I. (2002) The enzymology of prostaglandin endoperoxide H synthases-1 and -2. *Prostaglandins Other Lipid Mediat.*, **68-69,** 115-128.

26. Dietz, R., Nastainczyk, W., and Ruf, H.H. (1988) Higher oxidation states of prostaglandin H synthase. Rapid electronic spectroscopy detected two spectral intermediates during the peroxidase reaction with prostaglandin G_2. *Eur. J Biochem.,* **171,** 321-328.

27. Bambai, B. and Kulmacz, R.J. (2000) Prostaglandin H synthase. Effects of peroxidase cosubstrates on cyclooxygenase velocity. *J. Biol. Chem.*, **275,** 27608-27614.

28. Rogge, C.E., Liu, W., Wu, G., Wang, L.H., Kulmacz, R.J., and Tsai, A.L. (2004) Identification of Tyr504 as an alternative tyrosyl radical site in human prostaglandin H synthase-2. *Biochemistry*, **43,** 1560-1568.

29. Rogge, C.E., Ho, B., Liu, W., Kulmacz, R.J., and Tsai, A.L. (2006) Role of Tyr348 in Tyr385 radical dynamics and cyclooxygenase inhibitor interactions in prostaglandin H synthase-2. *Biochemistry*, **45,** 523-532.

30. Song, I., Ball, T.M., and Smith, W.L. (2001) Different suicide inactivation processes for the peroxidase and cyclooxygenase activities of

prostaglandin endoperoxide H synthase-1. *Biochem. Biophys. Res. Commun.*, **289,** 869-875.

31. Koshkin, V. and Dunford, H.B. (1999) Coupling of the peroxidase and cyclooxygenase reactions of prostaglandin H synthase. *Biochim. Biophys. Acta*, **1430,** 341-348.

32. Mizuno, K., Yamamoto, S., and Lands, W.E. (1982) Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. *Prostaglandins*, **23,** 743-757.

33. Kulmacz, R.J. and Wang, L.H. (1995) Comparison of hydroperoxide initiator requirements for the cyclooxygenase activities of prostaglandin H synthase-1 and -2. *J. Biol. Chem.*, **270,** 24019-24023.

34. Li, Y., Smith, T., Grabski, S., and DeWitt, D.L. (1998) The membrane association sequences of the prostaglandin endoperoxide synthases-1 and -2 isozymes. *J. Biol. Chem.*, **273,** 29830-29837.

35. Kulmacz, R.J. (1998) Cellular regulation of prostaglandin H synthase catalysis. *FEBS Lett.*, **430,** 154-157

36. Chen, W., Pawelek, T.R., and Kulmacz, R.J. (1999) Hydroperoxide dependence and cooperative cyclooxygenase kinetics in prostaglandin H synthase-1 and -2. *J. Biol. Chem.*, **274,** 20301-20306.

37. Yu, K., Bayona, W., Kallen, C.B., Harding, H.P., Ravera, C.P., McMahon, G., Brown, M., and Lazar, M.A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J. Biol. Chem.*, **270,** 23975-23983.

38. Smith, W.L., Garavito, R.M., and DeWitt, D.L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.*, **271,** 33157-33160.

39. Marnett, L.J. (2000) Cyclooxygenase mechanisms. *Curr. Opin. Chem. Biol.*, **4,** 545-552.

40. Kulmacz, R.J., Pendleton, R.B., and Lands, W.E. (1994) Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. Interpretation of reaction kinetics. *J. Biol. Chem.*, **269,** 5527-5536.

41. Barnett, J., Chow, J., Ives, D., Chiou, M., Mackenzie, R., Osen, E., Nguyen, B., Tsing, S., Bach, C., and Freire, J. (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim. Biophys. Acta*, **1209,** 130-139.

42. Laneuville, O., Breuer, D.K., DeWitt, D.L., Hla, T., Funk, C.D., and Smith, W.L. (1994) Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J Pharmacol. Exp. Ther.*, **271,** 927-934.

43. Tsai, A., Palmer, G., Xiao, G., Swinney, D.C., and Kulmacz, R.J. (1998) Structural characterization of arachidonyl radicals formed by prostaglandin H synthase-2 and prostaglandin H synthase-1 reconstituted with mangano protoporphyrin IX. *J. Biol. Chem.*, **273,** 3888-3894.

44. Hla, T., Bishop-Bailey, D., Liu, C.H., Schaefers, H.J., and Trifan, O.C. (1999) Cyclooxygenase-1 and -2 isoenzymes. *Int. J. Biochem.Cell Biol.,* **31,** 551-557.

45. O'Banion, M.K., Winn, V.D., and Young, D.A. (1992) cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. U. S. A.,* **89,** 4888-4892.

46. Xie, W.L., Chipman, J.G., Robertson, D.L., Erikson, R.L., and Simmons, D.L. (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U. S. A.,* **88,** 2692-2696.

47. Kujubu, D.A., Fletcher, B.S., Varnum, B.C., Lim, R.W., and Herschman, H.R. (1991) TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.*, **266,** 12866-12872.

48. Hla, T. and Neilson, K. (1992) Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U. S. A.,* **89,** 7384-7388.

49. Tanabe, T. and Tohnai, N. (2002) Cyclooxygenase isozymes and their gene structures and expression. *Prostaglandins Other Lipid Mediat.*, **68-69,** 95-114.

50. Appleby, S.B., Ristimaki, A., Neilson, K., Narko, K., and Hla, T. (1994) Structure of the human cyclooxygenase-2 gene. *Biochem. J.*, **302,** 723-727.

51. Hla, T., Ristimaki, A., Appleby, S., and Barriocanal, J.G. (1993) Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann. N. Y. Acad. Sci.*, **696,** 197-204.

52. Hansen, S.G., Strelow, L.I., Franchi, D.C., Anders, D.G., and Wong, S.W. (2003) Complete sequence and genomic analysis of rhesus cytomegalovirus. *J. Virol.*, **77,** 6620-6636.

53. Rue, C.A., Jarvis, M.A., Knoche, A.J., Meyers, H.L., DeFilippis, V.R., Hansen, S.G., Wagner, M., Fruh, K., Anders, D.G., Wong, S.W., Barry, P.A., and Nelson, J.A. (2004) A cyclooxygenase-2 homologue encoded by rhesus cytomegalovirus is a determinant for endothelial cell tropism. *J. Virol.*, **78,** 12529-12536.

54. Rivailler, P., Kaur, A., Johnson, R.P., and Wang, F. (2006) Genomic sequence of rhesus cytomegalovirus 180.92: insights into the coding potential of rhesus cytomegalovirus. *J. Virol.*, **80,** 4179-4182.

55. Garavito, R.M., Malkowski, M.G., and DeWitt, D.L. (2002) The structures of prostaglandin endoperoxide H synthases-1 and -2. *Prostaglandins Other Lipid Mediat.*, **68-69,** 129-152.

56. Loll, P.J., Picot, D., and Garavito, R.M. (1995) The Structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H_2 synthase. *Nat. Struct. Biol.*, **2,** 637-643.

57. Loll, P.J., Picot, D., Ekabo, O., and Garavito, R.M. (1996) Synthesis and use of iodinated nonsteroidal antiinflammatory drug analogs as crystallographic probes of the prostaglandin H_2 synthase cyclooxygenase active site. *Biochemistry*, **35,** 7330-7340.

58. Loll, P.J., Sharkey, C.T., O'Connor, S.J., Dooley, C.M., O'Brien, E., Devocelle, M., Nolan, K.B., Selinsky, B.S., and Fitzgerald, D.J. (2001) O-acetylsalicylhydroxamic acid, a novel acetylating inhibitor of prostaglandin H_2 synthase: Structural and functional characterization of enzyme-inhibitor interactions. *Mol. Pharmacol.*, **60,** 1407-1413.

59. Gupta, K., Selinsky, B.S., Kaub, C.J., Katz, A.K., and Loll, P.J. (2004) The 2.0 Å resolution crystal structure of prostaglandin H_2 synthase-1: Structural insights into an unusual peroxidase. *J. Mol. Biol.,* **335,** 503-518.

60. Gupta, K., Selinsky, B.S., and Loll, P.J. (2006) 2.0 Å Structure of prostaglandin H_2 synthase-1 reconstituted with a manganese porphyrin cofactor. *Acta Crystallogr. D. Biol. Crystallogr.*, **62,** 151-156.

61. Malkowski, M.G., Ginell, S.L., Smith, W.L., and Garavito, R.M. (2000) The productive conformation of arachidonic acid bound to prostaglandin synthase. *Science*, **289,** 1933-1937.

62. Malkowski, M.G., Thuresson, E.D., Lakkides, K.M., Rieke, C.J., Micielli, R., Smith, W.L., and Garavito, R.M. (2001) Structure of eicosapentaenoic and linoleic acids in the cyclooxygenase site of prostaglandin endoperoxide H synthase-1. *J. Biol. Chem.*, **276,** 37547-37555.

63. Thuresson, E.D., Malkowski, M.G., Lakkides, K.M., Rieke, C.J., Mulichak, A.M., Ginell, S.L., Garavito, R.M., and Smith, W.L. (2001) Mutational and x-ray crystallographic analysis of the interaction of dihomo-μ-linolenic acid with prostaglandin endoperoxide H synthases. *J. Biol. Chem.*, **276,** 10358-10365.

64. Kiefer, J.R., Pawlitz, J.L., Moreland, K.T., Stegeman, R.A., Hood, W.F., Gierse, J.K., Stevens, A.M., Goodwin, D.C., Rowlinson, S.W., Marnett, L.J., Stallings, W.C., and Kurumbail, R.G. (2000) Structural insights into the stereochemistry of the cyclooxygenase reaction. *Nature*, **405,** 97-101.

65. Otto, J.C., DeWitt, D.L., and Smith, W.L. (1993) N-glycosylation of prostaglandin endoperoxide synthases-1 and -2 and their orientations in the endoplasmic reticulum. *J. Biol. Chem.,* **268,** 18234-18242.

66. Percival, M.D., Bastien, L., Griffin, P.R., Kargman, S., Ouellet, M., and O'Neill, G.P. (1997) Investigation of human cyclooxygenase-2 glycosylation heterogeneity and protein expression in insect and mammalian cell expression systems. *Protein Expr. Purif.*, **9,** 388-398.

67. Nemeth, J.F., Hochgesang, G.P., Jr., Marnett, L.J., and Caprioli, R.M. (2001) Characterization of the glycosylation sites in cyclooxygenase-2 using mass spectrometry. *Biochemistry*, **40,** 3109-3116.

68. Gavel, Y. and von Heijne, G. (1990) Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Protein Eng.*, **3,** 433-442.

69. Wennogle, L.P., Liang, H., Quintavalla, J.C., Bowen, B.R., Wasvary, J., Miller, D.B., Allentoff, A., Boyer, W., Kelly, M., and Marshall, P. (1995) Comparison of recombinant cyclooxygenase-2 to native isoforms: aspirin labeling of the active site. *FEBS Lett.*, **371,** 315-320.

70. Sevigny, M.B., Li, C.F., Alas, M., and Hughes-Fulford, M. (2006) Glycosylation regulates turnover of cyclooxygenase-2. *FEBS Lett.,* **580**, 6533-6536.

71. Song, I. and Smith, W.L. (1996) C-terminal Ser/Pro-Thr-Glu-Leu tetrapeptides of prostaglandin endoperoxide H synthases-1 and -2 target the enzymes to the endoplasmic reticulum. *Arch. Biochem. Biophys.*, **334,** 67-72.

72. Garavito, R.M. and Mulichak, A.M. (2003) The structure of mammalian cyclooxygenases. *Annu. Rev. Biophys. Biomol. Struct.* **32,** 183-206.

73. Guo, Q. and Kulmacz, R.J. (2000) Distinct influences of carboxyl terminal segment structure on function in the two isoforms of prostaglandin H synthase. *Arch. Biochem. Biophys.*, **384,** 269-279.

74. Ren, Y., Loose-Mitchell, D.S., and Kulmacz, R.J. (1995) Prostaglandin H synthase-1: evaluation of C-terminus function. *Arch. Biochem. Biophys.*, **316,** 751-757.

75. Mbonye, U.R., Wada, M., Rieke, C.J., Tang, H.Y., DeWitt, D.L., and Smith, W.L. (2006) The 19 amino acid cassette of cyclooxygenase-2 mediates entry of the protein into the ER-associated degradation system. *J. Biol. Chem.,* **281**, 35770-35778.

76. Chandrasekharan, N.V., Dai, H., Roos, K.L., Evanson, N.K., Tomsik, J., Elton, T.S., and Simmons, D.L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl. Acad. Sci. U. S. A.*, **99**, 13926-13931.

77. Simmons, D.L., Botting, R.M., and Hla, T. (2004) Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol. Rev.*, **56,** 387-437.

78. Kulmacz, R.J., Van Der Donk, W.A., and Tsai, A.L. (2003) Comparison of the properties of prostaglandin H synthase-1 and -2. *Prog. Lipid Res.*, **42 ,** 377-404.

79. Hegde, R.S., Bernstein, H.D. (2006) The surprising complexity of signal sequences. *Trends Biochem. Sci.,* **31**, 563-571.

80. Martoglio, B. and Dobberstein, B. (1998) Signal sequences: more than just greasy peptides. *Trends Cell Biol.*, **8,** 410-415.

81. Campbell, I.D. and Bork, P. (1993) Epidermal growth factor-like modules. *Curr. Opin. Struct. Biol*. **3**, 385-392.

82. Appella, E., Weber, I.T., and Blasi, F. (1988) Structure and function of epidermal growth factor-like regions in proteins. *FEBS Lett.*, **231,** 1-4.

83. Garavito, R.M., Picot, D., and Loll, P.J. (1994) Prostaglandin H synthase. *Curr. Opin. Struct. Biol*., **4**, 529-535.

84. Picot, D. and Garavito, R.M. (1994) Prostaglandin H synthase: implications for membrane structure. *FEBS Lett.*, **346,** 21-25.

85. Kennedy, T.A., Smith, C.J., and Marnett, L.J. (1994) Investigation of the role of cysteines in catalysis by prostaglandin endoperoxide synthase. *J. Biol. Chem.*, **269,** 27357-27364.

86. Smith, C.J. and Marnett, L.J. (1996) Effects of cysteine-to-serine mutations on structural and functional properties of prostaglandin endoperoxide synthase. *Arch. Biochem. Biophys.*, **335,** 342-350.

87. Zeng, J. and Fenna, R.E. (1992) X-ray crystal structure of canine myeloperoxidase at 3 Å resolution. *J. Mol. Biol.*, **226,** 185-207.

88. Daiyasu, H. and Toh, H. (2000) Molecular evolution of the myeloperoxidase family. *J. Mol. Evol.*, **51,** 433-445.

89. Smith, T., McCracken, J., Shin, Y.K., and DeWitt, D. (2000) Arachidonic acid and nonsteroidal anti-inflammatory drugs induce conformational changes in the human prostaglandin endoperoxide H_2 synthase-2 (cyclooxygenase-2). *J. Biol. Chem.,* **275,** 40407-40415.

90. Yuan, C., Rieke, C.J., Rimon, G., Wingerd, B.A., and Smith, W.L. (2006) Partnering between monomers of cyclooxygenase-2 homodimers. *Proc. Natl. Acad. Sci. U. S. A.,* **103**, 6142-6147.

91. Liu, J., Seibold, S.A., Rieke, C.J., Song, I., Cukier, R.I., and Smith, W.L. (2007) Prostaglandin endoperoxide H synthases: Peroxidase hydroperoxide specificity and cyclooxygenase activation. *J. Biol. Chem.,* **282**, 18233-18244.

Seibold, S.A., Smith, W.L., and Cukier, R.I. (2004) Peroxidase Site of Prostaglandin Endoperoxide H Synthase-1: Docking and molecular dynamics studies with a prostaglandin endoperoxide analog. *J. Phys. Chem.,* **108**, 9297-9205.

92. Garavito, R.M., Picot, D., and Loll, P.J. (1995) The 3.1 Å X-ray crystal structure of the integral membrane enzyme prostaglandin H_2 synthase-1. *Adv. Prostaglandin Thromboxane Leukot. Res.*, **23,** 99-103.

93. Spencer, A.G., Thuresson, E., Otto, J.C., Song, I., Smith, T., DeWitt, D.L., Garavito, R.M., and Smith, W.L. (1999) The membrane binding domains of prostaglandin endoperoxide H synthases 1 and 2. Peptide mapping and mutational analysis. *J. Biol. Chem.*, **274,** 32936-32942.

94. Chubb, A.J., Fitzgerald, D.J., Nolan, K.B., and Moman, E. (2006) The productive conformation of prostaglandin G_2 at the peroxidase site of prostaglandin endoperoxide H synthase: docking, molecular dynamics, and site-directed mutagenesis studies. *Biochemistry*, **45,** 811-820.

95. Landino, L.M. and Marnett, L.J. (1996) Mechanism of hydroperoxide reduction by mangano-prostaglandin endoperoxide synthase. *Biochemistry*, **35,** 2637-2643.

96. Shimokawa, T. and Smith, W.L. (1991) Essential histidines of prostaglandin endoperoxide synthase. His-309 is involved in heme binding. *J. Biol. Chem.*, **266,** 6168-6173.

97. Seibold, S.A., Cerda, J.F., Mulichak, A.M., Song, I., Garavito, R.M., Arakawa, T., Smith, W.L., and Babcock, G.T. (2000) Peroxidase activity in prostaglandin endoperoxide H synthase-1 occurs with a neutral histidine proximal heme ligand. *Biochemistry*, **39,** 6616-6624.

98. Goodwin, D.C., Rowlinson, S.W., and Marnett, L.J. (2000) Substitution of tyrosine for the proximal histidine ligand to the heme of prostaglandin

endoperoxide synthase 2: implications for the mechanism of cyclooxygenase activation and catalysis. *Biochemistry*, **39,** 5422-5432.

99. Landino, L.M., Crews, B.C., Gierse, J.K., Hauser, S.D., and Marnett, L.J. (1997) Mutational analysis of the role of the distal histidine and glutamine residues of prostaglandin-endoperoxide synthase-2 in peroxidase catalysis, hydroperoxide reduction, and cyclooxygenase activation. *J. Biol. Chem.*, **272,** 21565-21574.

100. Ichimura, S., Uchida, T., Taniguchi, S., Hira, S., Tosha, T., Morishima, I., Kitagawa, T., and Ishimori, K. (2007) Unique peroxidase reaction mechanism in prostaglandin endoperoxidase H synthase-2: Compound I in prostaglandin endoperoxidase H synthase-2 can be formed without assistance by distal Gln residue. *J. Biol. Chem.,* **282**,16681-16690.

101. Bambai, B., Rogge, C.E., Stec, B., and Kulmacz, R.J. (2003) Role of Asn382 and Thr383 in activation and inactivation of human prostaglandin H synthase cyclooxygenase catalysis. *J. Biol. Chem.,* **27**9, 4084-4092

102. Thuresson, E.D., Lakkides, K.M., Rieke, C.J., Sun, Y., Wingerd, B.A., Micielli, R., Mulichak, A.M., Malkowski, M.G., Garavito, R.M., and Smith, W.L. (2001) Prostaglandin endoperoxide H synthase-1: The functions of cyclooxygenase active site residues in the binding, positioning and oxygenation of arachidonic acid. *J. Biol. Chem.*, **276,** 10347-10359.

103. Shimokawa, T. and Smith, W.L. (1992) Prostaglandin endoperoxide synthase. The aspirin acetylation region. *J. Biol. Chem.*, **267,** 12387-12392.

104. Schneider, C., Boeglin, W.E., Prusakiewicz, J.J., Rowlinson, S.W., Marnett, L.J., Samel, N., and Brash, A.R. (2001) Control of prostaglandin stereochemistry at the 15-carbon by cyclooxygenases-1 and -2. A critical role for serine 530 and valine 349. *J. Biol. Chem.*, **277,** 478-485.

105. Rowlinson, S.W., Crews, B.C., Lanzo, C.A., and Marnett, L.J. (1999) The binding of arachidonic acid in the cyclooxygenase active site of mouse prostaglandin endoperoxide synthase-2 (COX-2). A putative L-shaped binding conformation utilizing the top channel region. *J. Biol. Chem.*, **274,** 23305-23310.

106. Rieke, C.J., Mulichak, A.M., Garavito, R.M., and Smith, W.L. (1999) The role of arginine 120 of human prostaglandin endoperoxide H synthase-2 in the interaction with fatty acid substrates and inhibitors. *J. Biol. Chem.*, **274,** 17109-17114.

107. Mancini, J.A., Riendeau, D., Falgueyret, J.P., Vickers, P.J., and O'Neill, G.P. (1995) Arginine 120 of prostaglandin G/H synthase-1 is required for the inhibition by nonsteroidal anti-inflammatory drugs containing a carboxylic acid moiety. *J. Biol. Chem.*, **270,** 29372-29377.

108. Bhattacharyya, D.K., Lecomte, M., Rieke, C.J., Garavito, M., and Smith, W.L. (1996) Involvement of arginine 120, glutamate 524, and tyrosine 355 in the binding of arachidonate and 2-phenylpropionic acid inhibitors to the cyclooxygenase active site of ovine prostaglandin endoperoxide H synthase-1. *J. Biol. Chem.*, **271,** 2179-2184.

109. Greig, G.M., Francis, D.A., Falgueyret, J.P., Ouellet, M., Percival, M.D., Roy, P., Bayly, C., Mancini, J.A., and O'Neill, G.P. (1997) The interaction of arginine 106 of human prostaglandin G/H synthase-2 with inhibitors is not a universal component of inhibition mediated by nonsteroidal anti-inflammatory drugs. *Mol. Pharmacol.*, **52,** 829-838.

110. Kozak, K.R., Prusakiewicz, J.J., Rowlinson, S.W., Schneider, C., and Marnett, L.J. (2001) Amino acid determinants in cyclooxygenase-2 oxygenation of the endocannabinoid 2-arachidonylglycerol. *J. Biol. Chem.*, **276**, 30072-30077.

111. Yu, M., Ives, D., and Ramesha, C.S. (1997) Synthesis of prostaglandin E_2 ethanolamide from anandamide by cyclooxygenase-2. *J. Biol. Chem.*, **272,** 21181-21186.

112. So, O.Y., Scarafia, L.E., Mak, A.Y., Callan, O.H., and Swinney, D.C. (1998) The dynamics of prostaglandin H synthases. Studies with prostaglandin H synthase-2 Y355F unmask mechanisms of time-dependent inhibition and allosteric activation. *J. Biol. Chem.*, **273,** 5801-5807.

113. Kalgutkar, A.S., Crews, B.C., Rowlinson, S.W., Marnett, A.B., Kozak, K.R., Remmel, R.P., and Marnett, L.J. (2000) Biochemically based design of cyclooxygenase-2 (COX-2) inhibitors: facile conversion of nonsteroidal antiinflammatory drugs to potent and highly selective COX-2 inhibitors. *Proc. Natl. Acad. Sci. U. S. A.,* **97,** 925-930.

114. Timofeevski, S.L., Prusakiewicz, J.J., Rouzer, C.A., and Marnett, L.J. (2002) Isoform-selective interaction of cyclooxygenase-2 with indomethacin amides studied by real-time fluorescence, inhibition kinetics, and site-directed mutagenesis. *Biochemistry*, **41,** 9654-9662.

115. Prusakiewicz, J.J., Kingsley, P.J., Kozak, K.R., and Marnett, L.J. (2002) Selective oxygenation of N-arachidonylglycine by cyclooxygenase-2. *Biochem. Biophys. Res. Commun.*, **296,** 612-617.

116. Lecomte, M., Laneuville, O., Ji, C., DeWitt, D.L., and Smith, W.L. (1994) Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.*, **269,** 13207-13215.

117. DeWitt, D.L., el Harith, E.A., Kraemer, S.A., Andrews, M.J., Yao, E.F., Armstrong, R.L., and Smith, W.L. (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.*, **265,** 5192-5198.

118. Mancini, J.A., O'Neill, G.P., Bayly, C., and Vickers, P.J. (1994) Mutation of serine-516 in human prostaglandin G/H synthase-2 to methionine or aspirin acetylation of this residue stimulates 15-R-HETE synthesis. *FEBS Lett.*, **342,** 33-37.

119. Rowlinson, S.W., Kiefer, J.R., Prusakiewicz, J., Pawlitz, J.L., Kozak, K.R., Kalgutkar, A.S., Stallings, W.C., Kurumbail, R.G., and Marnett, L.J. (2003) A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385. *J. Biol. Chem.,* **278**, 45763-45769.

120. Hochgesang G.P., Nemeth-Cawley, J.F., Rowlinson, S.W., Caprioli, R.M., and Marnett, L.J. (2003) Functional analysis of the molecular

determinants of cyclooxygenase-2 acetylation by 2-acetoxyphenylhept-2-ynyl sulfide. *Arch. Biochem. Biophys.*, **409,** 127-133.

121. Hochgesang, G.P., Jr., Rowlinson, S., and Marnett, L.J. (2000) Tyrosine-385 is critical for acetylation of cyclooxygenase-2 by aspirin. *J. Am. Chem. Soc*. **122**, 6514-6515.

122. Thuresson, E.D., Lakkides, K.M., and Smith, W.L. (2000) Different catalytically competent arrangements of arachidonic acid within the cyclooxygenase active site of prostaglandin endoperoxide H synthase-1 lead to the formation of different oxygenated products. *J. Biol. Chem.*, **275,** 8501-8507.

123. Hsi, L.C.; Tsai, A.L.; Kulmacz, R.J.; English, D.G.; Siefker, A.O.; Otto, J.C.; Smith, W.L. (1993) Trp387 and the putative leucine zippers of PGH synthases-1 and –2. *J. Lipid Mediat.,* **6**, 131-138

124. Harman, C.A., Rieke, C.J., Garavito, R.M., and Smith, W.L. (2004) Crystal structure of arachidonic acid bound to a mutant of prostaglandin endoperoxide H synthase-1 that forms predominantly 11-HPETE. *J. Biol. Chem.,* **279**, 42929-35.

125. Rowlinson, S.W., Crews, B.C., Goodwin, D.C., Schneider, C., Gierse, J.K., and Marnett, L.J. (2000) Spatial requirements for 15-(R)-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid synthesis within the cyclooxygenase active site of murine COX-2. Why acetylated COX-1 does not synthesize 15-(R)-HETE. *J. Biol. Chem.*, **275,** 6586-6591.

126. Wong, E., Bayly, C., Waterman, H.L., Riendeau, D., and Mancini, J.A. (1997) Conversion of prostaglandin G/H synthase-1 into an enzyme sensitive to PGHS-2-selective inhibitors by a double His513Arg and Ile523Val mutation. *J. Biol. Chem.*, **272,** 9280-9286.

127. Grosser, T., Yusuff, S., Cheskis, E., Pack, M.A., and FitzGerald, G.A. (2002) Developmental expression of functional cyclooxygenases in zebrafish. *Proc. Natl. Acad. Sci. U. S. A.,* **99**, 8418-8423

128. Roberts, S.B., Langenau, D.M., and Goetz, F.W. (2000) Cloning and characterization of prostaglandin endoperoxide synthase-1 and -2 from the brook trout ovary. *Mol. Cell Endocrinol.*, **160,** 89-97.

129. Klein, S.L., Strausberg, R.L., Wagner, L., Pontius, J., Clifton, S.W., and Richardson, P. (2002) Genetic and genomic tools for Xenopus research: The NIH Xenopus initiative. *Dev. Dyn.*, **225,** 384-391.

130. Kozak, K.R., Prusakiewicz, J.J., Rowlinson, S.W., Prudhomme, D.R., and Marnett, L.J. (2003) Amino acid determinants in cyclooxygenase-2 oxygenation of the endocannabinoid anandamide. *Biochemistry*, **42,** 9041-9049.

131. Schneider, C., Boeglin, W.E., and Brash, A.R. (2004) Identification of two cyclooxygenase active site residues, leucine-384 and glycine-526, that control carbon ring cyclization in prostaglandin biosynthesis. *J. Biol. Chem.,* **279**, 4404-4414.

132. Guo, Q., Chang, S., Diekman, L., Xiao, G., and Kulmacz, R.J. (1997) Comparison of prostaglandin H synthase isoform structures using limited proteolytic digestion. *Arch. Biochem. Biophys.*, **344,** 150-158.

133. Laneuville, O., Breuer, D.K., Xu, N., Huang, Z.H., Gage, D.A., Watson, J.T., Lagarde, M., DeWitt, D.L., and Smith, W.L. (1995) Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)- octadecatrienoic acids from α-linolenic acid. *J. Biol. Chem.*, **270,** 19330-19336.

134. Kozak, K.R., Rowlinson, S.W., and Marnett, L.J. (2000) Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. *J. Biol. Chem.*, **275,** 33744-33749.

135. Kozak, K.R., Crews, B.C., Ray, J.L., Tai, H.H., Morrow, J.D., and Marnett, L.J. (2001) Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides in vitro and in vivo. *J. Biol. Chem.,* 276, 36993-36998.

136. Kozak, K.R., Crews, B.C., Morrow, J.D., Wang, L.H., Ma, Y.H., Weinander, R., Jakobsson, P.J., and Marnett, L.J. (2002) Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J. Biol. Chem.,* **277**, 44877-85.

137. Rouzer, C.A. and Marnett, L.J. (2005) Structural and functional differences between cyclooxygenases: Fatty acid oxygenases with a critical role in cell signaling. *Biochem. Biophys. Res. Commun.,* **338**, 34-44.

138. Vane, J.R. and Warner, T.D. (2000) Nomenclature for COX-2 inhibitors. *Lancet*, **356,** 1373-1374.

139. Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New. Biol.*, **231,** 232-235.

140. Tsai, A.L., Palmer, G., and Kulmacz, R.J. (1992) Prostaglandin H synthase. Kinetics of tyrosyl radical formation and of cyclooxygenase catalysis. *J. Biol. Chem.*, **267,** 17753-17759.

141. Claria, J. and Serhan, C.N. (1995) Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. U. S. A.,* **92,** 9475-9479.

142. Serhan, C.N. and Oliw, E. (2001) Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways. *J Clin. Invest.*, **107,** 1481-1489.

143. Fiorucci, S., Distrutti, E., de Lima, O.M., Romano, M., Mencarelli, A., Barbanti, M., Palazzini, E., Morelli, A., and Wallace, J.L. (2003) Relative contribution of acetylated cyclooxygenase (COX)-2 and 5-lipooxygenase (LOX) in regulating gastric mucosal integrity and adaptation to aspirin. *FASEB J.*, **17,** 1171-1173.

144. Dannhardt, G. and Kiefer, W. (2001) Cyclooxygenase inhibitors-current status and future prospects. *Eur. J. Med. Chem.*, **36,** 109-126.

145. Marnett, L.J. (2002) Recent developments in cyclooxygenase inhibition. *Prostaglandins Other Lipid Mediat.*, **68-69,** 153-164.

146. Grosser, T., Fries, S., and FitzGerald, G.A. (2006) Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J. Clin. Invest.*, **116,** 4-15.

147. Warner, T.D. and Mitchell, J.A. (2004) Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. *FASEB J.*, **18,** 790-804.

148. Ouellet, M. and Percival, M.D. (2001) Mechanism of acetaminophen inhibition of cyclooxygenase isoforms. *Arch. Biochem. Biophys.*, **387,** 273-280.

149. Lucas, R., Warner, T.D., Vojnovic, I., and Mitchell, J.A. (2005) Cellular mechanisms of acetaminophen: role of cyclooxygenase. *FASEB J.*, **19,** 635-637.

150. Boutaud, O., Aronoff, D.M., Richardson, J.H., Marnett, L.J., and Oates, J.A. (2002) Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H_2 synthases. *Proc. Natl. Acad. Sci. U. S. A.,* **99,** 7130-7135.

151. Schneider, C.; Boeglin, W.E.; Brash, A.R.(2005) Human cyclooxygenase-1 and an alternative splice variant: Contrasts in expression of mRNA, protein, and catalytic activities. *Biochem. J.*, **385,** 57-64

152. Mitchell, J.A. and Warner, T.D. (2006) COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. *Nat. Rev. Drug Discov.*, **5,** 75-86.

153. Snipes, J.A., Kis, B., Shelness, G.S., Hewett, J.A., and Busija, D.W. (2005) Cloning and characterization of cyclooxygenase-1b (putative COX-3) in Rat. *J. Pharmacol. Exp. Ther*. **313**, 668-676.

154. Kis, B., Snipes, J.A., Gaspar, T., Lenzser, G., Tulbert, C.D., and Busija, D.W. (2006) Cloning of cyclooxygenase-1b (putative COX-3) in mouse. *Inflamm. Res*, **55,** 274-278.

155. Qin, N., Zhang, S.P., Reitz, T.L., Mei, J.M., and Flores, C.M. (2005) Cloning, expression and functional characterization of human COX-1 splicing variants: evidence for intron 1 retention. *J. Pharmacol. Exp. Ther*., **315**, 1298-1305.

156. Warner, T.D., Vojnovic, I., Giuliano, F., Jiminez, R., Bishop-Bailey, D., and Mitchell, J.A. (2004) Cyclooxygenases-1, 2 and 3 and the production of prostaglandin I_2: investigating the activities of acetaminophen and cyclooxygenase-2-selective inhibitors in rat tissues. *J. Pharmacol. Exp. Ther*. **310**, 642-647.

157. Dinchuk, J.E., Liu, R.Q., and Trzaskos, J.M. (2003) COX-3: in the wrong frame in mind. *Immunol. Lett.*, **86,** 121.

158. Kis, B., Snipes, J.A., Isse, T., Nagy, K., and Busija, D.W. (2003) Putative cyclooxygenase-3 expression in rat brain cells. *J. Cereb. Blood Flow Metab.*, **23,** 1287-1292.

159. Aronoff, D.M., Oates, J.A., and Boutaud, O. (2006) New insights into the mechanism of action of acetaminophen: Its clinical pharmacologic characteristics reflect its inhibition of the two prostaglandin H_2 synthases. *Clin. Pharmacol.Ther.*, **79,** 9-19.

160. Lands, W.E. and Hanel, A.M. (1982) Phenolic anticyclooxygenase agents in antiinflammatory and analgesic therapy. *Prostaglandins*, **24,** 271-277.

161. Stanley, D. (2006) Prostaglandins and other eicosanoids in insects: biological significance. *Annu. Rev. Entomol.*, **51,** 25-44.

162. Stanley, D. and Miller, J.S. (2006) Eicosanoid actions in insect cellular immune functions. *Entomologia Experimentalis et Applicata,* **19**, 1-13.

163. Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., etc. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science*, **298,** 2157-2167.

164. Järving, R., Järving, I., Kurg, R., Brash, A.R., and Samel, N. (2004) On the evolutionary origin of cyclooxygenase isozymes: Characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages. *J. Biol. Chem.,* **279**, 13624-33.

165. Qian, Y., Yuan, J., Essenberg, R.C., Bowman, A.S., Shook, A.L., Dillwith, J.W., Sauer, J.R. (1998) Prostaglandin E_2 in the salivary glands of the female tick, Amblyomma americanum (L.): calcium mobilization and exocytosis. *Insect Biochem. Mol.Biol*., **28,** 221-228

166. Weinheimer, A.J. and Spraggins, R.L. (1969) The occurrence of two new prostaglandin derivatives (15-epi-PGA_2 and its acetate, methyl ester) in the gorgonian *Plexaura homomalla* chemistry of coelenterates. XV. *Tetrahedron Lett.*, **59,** 5185-5188.

167. Gerwick, W.H., Nagle, D.G., and Proteau, P.J. (1993) Oxylipins from marine invertebrates. *Top. Curr. Chem.,* **167**, 117-180.

168. Weinheimer, A.J. Prostaglandins from *Plexaura homomalla*: Ecology, utilization and conservation of a major medical marine resource. (Bayer, F.M., and Weinheimer, A.J., Eds.), Univ.of Miami Press, Coral Gables, FL . 1973.

169. Coll, J.C. The Chemistry and chemical ecology of octocorals (Coelenterata, Anthozoa, Octocorallia). (1992) *Chem. Rev*., **92**, 613-631.

170. Song, W.C. and Brash, A.R. (1991) Investigation of the allene oxide pathway in the coral *Plexaura homomalla*: formation of novel ketols and isomers of prostaglandin A_2 from 15-hydroxyeicosatetraenoic acid. *Arch. Biochem. Biophys.*, **290,** 427-435.

171. Schneider, W.P., Bundy, G.L., Lincoln, F.H., Daniels, E.G., and Pike, J.E. (1977) Isolation and chemical conversions of prostaglandins from *Plexaura homomalla*: Preparation of prostaglandin E_2, prostaglandin $F_{2\alpha}$, and their 5, 6-trans isomers. *J. Am. Chem. Soc.*, **99,** 1222-1232.

172. Bundy, G.L. (1985) Nonmammalian sources of eicosanoids. *Adv. Prostaglandin Thromboxane Leukot. Res.*, **14,** 229-262.

173. Light, R.J. and Samuelsson, B. (1972) Identification of prostaglandins in the gorgonian, *Plexaura homomalla*. *Eur. J. Biochem.*, **28,** 232-240.

174. Corey, E.J. and Washburn, W.N. (1974) The role of the symbiotic algae of *Plexaura homomalla* in prostaglandin biosynthesis. *J. Am. Chem. Soc.*, **96,** 934-935.

175. Corey, E.J., Ensley, H., Hamberg, M., and Samuelsson, B. (1975) Disparate pathways of prostaglandin biosynthesis in coral and mammalian systems. *J. C. S. Chem. Comm*., 277-278.

176. Corey, E.J., Washburn, W.N., and Chen, J.C. (1973) Studies on the prostaglandin A_2 synthetase complex from *Plexaura homomalla*. *J. Am. Chem. Soc.*, **95,** 2054-2055.

177. Brash, A.R., Baertschi, S.W., Ingram, C.D., and Harris, T.M. (1987) On non-cyclooxygenase prostaglandin synthesis in the sea whip coral, *Plexaura homomalla*: an 8(R)-lipoxygenase pathway leads to formation of an α-ketol and a racemic prostanoid. *J. Biol. Chem.*, **262,** 15829-15839.

178. Koljak, R., Boutaud, O., Shieh, B.H., Samel, N., and Brash, A.R. (1997) Identification of a naturally occurring peroxidase-lipoxygenase fusion protein. *Science*, **277,** 1994-1996.

179. Blee, E. (1998) Phytooxylipins and plant defense reactions. *Prog. Lipid Res.*, **37,** 33-72.

180. Feussner, I. and Wasternack, C. (2002) The lipoxygenase pathway. *Annu. Rev. Plant Biol.,* **53,** 275-297.

181. Boutaud, O. and Brash, A.R. (1999) Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral *Plexaura homomalla*. *J. Biol. Chem.*, **274,** 33764-33770.

182. Oldham, M.L., Brash, A.R., and Newcomer, M.E. (2005) The structure of coral allene oxide synthase reveals a catalase adapted for metabolism of a fatty acid hydroperoxide. *Proc. Natl. Acad. Sci. U. S. A.,* **102,** 297-302.

183. Varvas, K., Järving, I., Koljak, R., Valmsen, K., Brash, A.R., and Samel, N. (1999) Evidence of a cyclooxygenase-related prostaglandin synthesis in coral. The allene oxide pathway is not involved in prostaglandin biosynthesis. *J. Biol. Chem.*, **274,** 9923-9929.

184. Varvas, K., Järving, I., Koljak, R., Vahemets, A., Pehk, T., Müürisepp, A.M., Lille, Ü., and Samel, N. (1993) *Tetrahedron Lett*., 34, 3643-3646.

185. Varvas, K., Koljak, R., Järving, I., Pehk, T., and Samel, N. (1994) Endoperoxide pathway in prostaglandin biosynthesis in the soft coral *Gersemia fruticosa*. *Tetrahedron Lett.*, 35, 8267-8270.

186. Koljak, R., Järving, I., Kurg, R., Boeglin, W.E., Varvas, K., Valmsen, K., Ustav, M., Brash, A.R., and Samel, N. (2000) The basis of prostaglandin synthesis in coral: cloning and expression of a cyclooxygenase from the arctic soft coral *Gersemia fruticosa*. *J. Biol. Chem.*, **276,** 7033-7040.

187. Schneider, W.P., Hamilton, R.D., and Rhuland, L.E. (1972) Occurrence of Esters of 15S-Prostaglandin A_2 and E_2 in Coral. *J. Am. Chem. Soc*., 94, 2122-2123.

188. Zou, J., Neumann, N.F., Holland, J.W., Belosevic, M., Cunningham, C., Secombes, C.J., and Rowley, A.F. (1999) Fish macrophages express a cyclooxygenase-2 homologue after activation. *BioChem. J*, **340,** 153-159.

189. Yang, T., Forrest, S.J., Stine, N., Endo, Y., Pasumarthy, A., Castrop, H., Aller, S., Forrest, J.N., Jr., Schnermann, J., and Briggs, J. (2002) Cyclooxygenase cloning in dogfish shark, *Squalus acanthias*, and its role in rectal gland Cl secretion. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **283,** 631-R637.

190. Ishikawa, T.O., Griffin, K.J., Banerjee, U., and Herschman, H.R. (2007) The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. *Biochem. Biophys. Res. Commun.*, **352,** 181-187.

191. Parve, O., Järving, I., Martin, I., Metsala, A., Vallikivi, I., Aidnik, M., Pehk, T., and Samel, N. (1999) Lipase-catalysed acylation of prostanoids. *Bioorg. Med. Chem. Lett.*, **9,** 1853-1858.

192. Schneider, W.P., Morge, R.A., and Henson, B.E. (1977) The occurrence of 13, 14-dihydro and 13, 14-cis-unsaturated prostaglandins in the coral, *Plexaura homomalla*. Synthesis of 13, 14-cis-prostaglandin E_2, 15-acetate methyl ester, and the 13, 14-cis analogues of prostaglandin $F_{2}\alpha$ and prostaglandin $F_{2\beta}$. *J. Am. Chem. Soc.*, **99,** 6062-6066.

193. Garavito, R.M. and White, S.H. (1997) Membrane proteins. Structure, assembly, and function: a panoply of progress. *Curr. Opin. Struct. Biol.,* **7,** 533-536.

194. Gierse, J.K., Hauser, S.D., Creely, D.P., Koboldt, C., Rangwala, S.H., Isakson, P.C., and Seibert, K. (1995) Expression and selective inhibition of the constitutive and inducible forms of human cyclooxygenase. *Biochem. J.*, **305,** 479-484.

195. Shimokawa, T. and Smith, W.L. (1992) Expression of prostaglandin endoperoxide synthase-1 in a baculovirus system. *Biochem. Biophys. Res. Commun*, **183,** 975-982.

196. Nakano, J. (1972) Relationship between the chemical structure of prostaglandins and their vasoactivities in dogs. *Br. J. Pharmacol.*, **44,** 63-70.

197. Yilmaz, E. (2001) Biotechnological production of prostaglandin. *Biotechnol. Adv.*., **19,** 387-397.

198. Nakano, J., Anggard, E., and Samuelsson, B. (1969) 15-Hydroxy-prostanoate dehydrogenase. Prostaglandins as substrates and inhibitors. *Eur. J Biochem.,* **11,** 386-389.

199. Mikaelian, I., and Sergeant, A. (1992) A general and fast method to generate multiple site directed mutations. *Nucleic Acids Res*. **20**, 376.

200. Henikoff, S. and Henikoff, J.G. (1992) Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. U. S. A.,* **89,** 10915-10919.

ABBREVIATIONS

AA	arachidonic acid (eicosatetraenoic acid)
AG	arachidonylglycerol
AEA	arachidonylethanolamide (anandamide)
AOS	allene oxide synthase
ApAP	acetaminophen
ATL	aspirin-triggered lipoxin
COX	cyclooxygenase
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERAD	ER associated degradation
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
KETE	ketoeicosatetraenoic acid
LOX	lipoxygenase
MBD	membrane binding domain
moi	multiplicity of infection
NSAID	nonsteroidal anti-inflammatory drug
PG	prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
PCR	polymerase chain reaction
RP-HPLC	reversed-phase HPLC
SP-HPLC	straight-phase HPLC
TxA	thromboxane

SUMMARY

Prostaglandins are a family of chemical messengers which participate in a variety of physiological mechanisms, therefore playing critical roles in health and disease states.

The bifunctional enzyme cyclooxygenase (COX) catalyses the conversion of arachidonic acid, via a free radical mechanism, into prostaglandin H_2 (PGH_2). The COX enzyme actually performs two sequential reactions: a cyclooxygenase reaction, which converts arachidonic acid to PGG_2, followed by a peroxidase reaction, which converts PGG_2 to PGH_2. During the reactions five chiral centres are introduced into an achiral arachidonic acid. The correct stereochemistry of prostaglandins is a prerequisite of their biological activity and is therefore under a strict enzymatic control.

Two COX isoforms are known. COX-1, which is constitutively expressed in most tissues, is responsible for the physiological production of prostaglandins; and COX-2, which is induced by cytokines, mitogens and endotoxin in inflammatory cells, is responsible for the elevated production of prostaglandins during inflammation. The COX isozymes are integral membrane enzymes which are found in the endoplasmic reticulum as homodimers. The tertiary and quaternary structures of COX isoforms are almost identical, consisting of three folding domains: an N-terminal epidermal growth factor-like domain, a membrane-binding domain, and a catalytical domain containing cyclooxygenase and peroxidase active sites.

The Caribbean Sea soft coral *Plexaura homomalla* is known as the richest natural source of prostaglandins (2-5% of dry weight). Depending on the location of the coral in the Caribbean, the carbon 15 of prostaglandins is in *S*- or *R*-configuration. In spite of intense studies the mechanism of prostaglandin formation has remained unresolved for several decades. All coral preparations used were unable to synthesise prostaglandins *in vitro*. It was proposed that differently from the mammalian endoperoxide pathway, corals metabolise arachidonic acid to an unstable allene epoxide, which is later converted to cyclic eicosanoids including prostaglandins. This type of pathway is known to be used in plants for jasmonic acid synthesis.

The aim of the present study was to elicit the pathway of prostaglandin synthesis in coral and to figure out the structural determinants of oxygenation stereospecificity.

We clearly established the occurrence of 15*R*-specific and 15*S*-specific COX-enzymes in variants of the same species of *P. homomalla*. We cloned and expressed two enzymes sharing a 97% amino acid identity, yet they perform the oxygenation reaction of arachidonic acid with an opposite stereoselectivity at carbon 15. With this finding, we demonstrated that a very minor sequence divergence accounts for the contents of epimeric prostaglandins in the two variants of coral and that the differences do not arise by isomerisation of the products.

Due to the extremely high sequence identity, the pair of enzymes served as an ideal model for determining the molecular basis of understanding how enzymes control oxygenation during catalysis. Based on the sequence information we cloned several mutant enzymes, analysed their reaction products and figured out that the oxygenation specificity at C-15 can be reversed by mutagenesis.

Our results show that the main stereospecificity determinant is located in position 349 (valine in *S*-specific and isoleucine in *R*-specific COX). We also established that a full change in stereochemistry at the carbon 15 of prostaglandins relates to five amino acid substitutions on helices 5 and 6 of the coral COX. In COX proteins, these helices form a helix-turn-helix motif that traverses through the entire protein, contributing to the second shell of residues around the oxygenase active site; it constitutes the most highly conserved region where even slight changes result in a loss of catalytic activity. The finding that this region is the least conserved region between *P. homomalla* 15*S*-specific and 15*R*-specific COX further supports the significance of helices 5 and 6 in maintaining the 15*S* -configuration in the prostaglandin products.

KOKKUVÕTE

Prostaglandiinid on hormoonilaadsed signaalmolekulid, mis osalevad paljude erinevate füsioloogiliste ja patofüsioloogiliste protsesside regulatsioonil. Prostaglandiinide sünteesi võtmeensüümideks selgroogsetes organismides on tsüklooksügenaasid (COX), mis katalüüsivad membraanist vabastatud arahhidoonhappe oksüdeerimist hüdroperoksü-endoperoksiidiks (PGG_2) ja selle hilisemat taandamist hüdroksü-endoperoksiidiks (PGH_2). Reaktsiooni käigus muudetakse akiraalne arahhidoonhape viie sümmeetriatsentriga molekuliks, üheks kolmekümne kahest võimalikust stereoisomeerist. Signaalmolekulide õige käelisus on nende bioloogilise aktiivsuse eelduseks ja seetõttu range ensümaatilise kontrolli all.

Kuni 90-ndate alguseni tunti ainult üht COX isovormi. Nüüdseks on tõestatud kahe isosüümi olemasolu, millest konstitutiivset COX-1 seostatakse rohkem füsioloogilise regulatsiooniga, indutseeritavat COX-2 aga põletikuliste protsesside ja vähkkasvajate tekkega. Aspiriini ja teiste mittesteroidsete põletikuvastaste vahendite efekt seisneb prostaglandiinide sünteesi inhibeerimises. Enamus esimese ja teise põlvkonna inhibiitoreid on mitteselektiivsed, omades rohkelt negatiivseid kõrvalmõjusid. COX isosüümide katalüüsi mehhanismide ja regulatsiooni tundmine võimaldab ensüümi selektiivselt inhibeerida ja seega vähendada mittesoovitavaid efekte.

COX ensüümid on bifunktsionaalsed, glükosüleeritud, endoplasmaatilises retiikulumis lokaliseeruvad integraalsed membraanvalgud molekulmassiga 67-72 kDa. Lahuses ja samuti membraanis esinevad nad homodimeerina. Valgu monomeer koosneb kolmest domeenist : N-terminaalsest EGF-laadsest domeenist, monotoopsest membraan-

seostumisdomeenist ja suurest peamiselt heeliksitest koosnevast kahe aktiivtsentriga katalüütilisest domeenist.

Kariibi mere korall *Plexaura homomalla* on kõige rikkam looduslik prostaglandiinide allikas, sisaldades neid kuni 5% kuivainest. Prostaglandiine sünteesivat valku pole senini õnnestunud detekteerida, kuna vastavatel ensüümpreparaatidel puudub prostaglandiinide sünteesi aktiivsus. *In vitro* tingimustes metaboliseerivad korallipreparaadid eksogeense arahhidoonhappe alleenoksiidiks, mida on peetud vaheühendiks tsükliliste eikosanoidide biosünteesil. Seda tüüpi reaktsioonid toimuvad taimedes jasmoonhappe sünteesil lipoksügenaasi ja alleenoksiidsüntetaasi toimel. *P. homomalla* on eriline veel selle poolest, et sõltuvalt kasvukohast sisaldab korall prostaglandiine, mille viieteistkümnenda asendi süsinik on kas *S*- või *R*-konfiguratsioonis. Bioloogiliselt väheaktiivsete 15*R* prostaglandiinide olemasolu ei ole senini üheski teises organismis kirjeldatud.

Käesoleva töö eesmärgiks seati prostaglandiinide biosünteesiraja väljaselgitamine korallis *P. homomalla.* Erilist huvi pakkus 15*R* epimeeride tekkemehhanism. Töö raames kloneeriti ja ekspresseeriti korallist *P. homomalla* 15*R*- ja 15*S*- spetsiifilised tsüklooksügenaasid. Sellega näidati imetajatele omase sünteesiraja olemasolu selgrootus organismis. Tõestati, et 15*R*- prostaglandiinid ei ole tekkinud isomerisatsiooni tagajärjel, vaid neid sünteesib uudse stereospetsiifikaga tsüklooksügenaas. Saadud stereoisomeere sünteesivad ensüümid erinevad teineteisest seitsmeteistkümne aminohappe jäägi poolest. Suur primaarjärjestuste sarnasus võimaldas kindlaks määrata reaktsiooni stereospetsiifilisuse struktuurilised determinandid. Selgus, et kõige suuremat mõju omab 349 asendis paiknev aminohape (valiin 15*S*- COX ja isoleutsiin 15*R*-COX ensüümil). Produkti stereokeemia täielikuks pööramiseks on vajalik veel nelja aminohappe jäägi asendamine. Ülejäänud stereoisomeeride teket määravad aminohapped asuvad Val/Ile349 samal või sellega külgneval heeliksil (heeliksid 5 ja 6). Need kaks pikka heeliksit on tsüklooksügenaaside ja ka teiste peroksüdaaside peamiseks struktuurielemendiks, olles ühtlasi ka nende ensüümide kõige konserveerunum ala. Antud töös me tõestasime, et tegemist ei ole pelgalt valgu karkassi moodustava heeliksipaariga vaid prostaglandiinide õiget konfiguratsiooni tagava struktuurifragmendiga.