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**Prostaglandin Synthesis in
Marine Arthropods and Red Algae**

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been submitted for any other academic degree.

/Kristella Hansen/

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**Prostaglandiinide süntees
mere lüljalgsetes ja punavetikates**

KRISTELLA HANSEN

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INTRODUCTION

Cyclooxygenases (COXs) catalyse the first two steps in the biosynthesis of prostaglandins, which are well-known lipid mediators in vertebrates. They regulate many physiologically important processes, such as body temperature and sleep-wake mechanisms, smooth muscle contraction/relaxation, Na⁺ excretion and fertility, as well as various pathological events, such as inflammation, pain, fever and cancer.

There are several reports on the occurrence of prostaglandins in different arthropods. Their physiological roles have been mostly associated with reproduction, ion transport, immune system and defence reactions. However, to date, no COX-like enzymes have been identified in completely sequenced insect genomes of *Drosophila* sp. *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Tribolium castaneum* or others. But COX-like sequences have been found in some aquatic arthropods.

Prostaglandins have not been reliably detected and COXs have not been cloned or characterized in flowering plants. However, in red algae, several different prostaglandins have been identified. Food poisoning cases resulting in death due to the ingestion of the edible red alga *Gracilaria vermiculophylla* have been reported. They were thought to be caused by the large amounts of prostaglandins and the further increase in the concentration of prostaglandins in a COX reaction. A better understanding of the prostaglandin biosynthesis in red algae might help to avoid such situations in the future.

Initially, COXs were associated only with mammals, but were later also associated with other vertebrates, bony fish and birds. There has been speculation that some lower marine organisms may use an alternative lipoxygenase (LOX)-based route of prostaglandin synthesis. The research in our laboratory revealed that at least some marine invertebrates use a prostaglandin synthesis pathway similar to that of mammals: COXs from two soft corals were cloned and characterized. The aim of this study was to determine what kind of prostaglandin synthesis pathway is represented in other prostaglandin-containing lower marine organisms, more specifically in marine arthropods and red algae.

In this study, the first non-animal COXs from the red algae *G. vermiculophylla* and *Coccotylus truncatus* were cloned and characterized. The prostaglandin synthesis pathway in two amphipod crustaceans, *Gammarus* sp. and *Caprella* sp., was described even more comprehensively: the structure and function of the first arthropod COXs, as well as membrane-associated prostaglandin E synthases-2 (mPGES-2s), are described.

ORIGINAL PUBLICATIONS

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

Publication I

Varvas K., Kasvandik S., Hansen K., Järving I., Morell I., Samel N. (2013). Structural and catalytic insights into the algal prostaglandin H synthase reveal atypical features of the first non-animal cyclooxygenase. *Biochim. Biophys. Acta.* 1831: 863-871.

Publication II

Varvas K., Kurg R., Hansen K., Järving R., Järving I., Valmsen K., Löhelaid H., Samel N. (2009). Direct evidence of the cyclooxygenase pathway of prostaglandin synthesis in arthropods: genetic and biochemical characterization of two crustacean cyclooxygenases. *Insect Biochem. Mol. Biol.* 39: 851-860.

Publication III

Hansen K., Varvas K., Järving I., Samel N. (2014). Membrane-associated prostaglandin E synthase-2 from crustacean arthropods. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* 174: 45-52.

Author's contribution:

Publication I: The author participated in the planning, in the experimental work (except for the protein purification), in the data analysis and in the manuscript preparation.

Publication II: The author participated in the planning of experiments and carried out the experimental work (except for the COS-7 cell experiments), and participated in the data analysis and in the manuscript preparation.

Publication III: The author planned and performed the experimental work, analysed the data, and participated in the manuscript preparation.

ABBREVIATIONS

12-HHT	12(S)-hydroxy-5,8,10(Z,E,E)-heptadecatrienoic acid
2-AG	2- arachidonoylglycerol
AA	arachidonic acid
AEA	arachidonoyl ethanolamide
COX	cyclooxygenase
cox	cyclooxygenase active site
DGLA	dihomo- γ -linolenic acid
E _{allo}	allosteric subunit of the COX homodimer
E _{cat}	catalytic subunit of the COX homodimer
EGF	epidermal growth factor
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
GSH	glutathione
GST	glutathione S-transferase
LPS	lipopolysaccharide
LDS	linoleate diol synthase
MAPEG	Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism
MBD	membrane binding domain
MDA	malondialdehyde
MGST	microsomal glutathione S-transferase
MPO	myeloperoxidase
NSAIDs	non-steroidal anti-inflammatory drugs
PGE ₂	prostaglandin E ₂
PGES	prostaglandin E synthase
PGDS	prostaglandin D synthase
PGFS	prostaglandin F synthase
PLA ₂	phospholipase A ₂
pox	peroxidase active site
RP-HPLC	reversed-phase high-pressure liquid chromatography
RT-PCR	reverse transcriptase polymerase chain reaction
Pxt	peroxinectin
α -DOX	α -dioxygenase

1. REVIEW OF THE LITERATURE

1.1. Oxylipins as signalling molecules

Oxylipins constitute a large family of oxidized fatty acids and their derivatives. They are common in mammals and other organisms: flowering plants, mosses, algae, bacteria and fungi. In plants, oxylipins act as signalling molecules regulating developmental processes and mediating responses to biotic- and abiotic stresses. Unlike plants, in which 18 carbon fatty acids are dominant and also most often used for oxylipin synthesis, mammals predominantly use 20 carbon fatty acids (Andreou et al., 2009; Brodhun and Feussner, 2011). Eicosanoids, which are derived from 20 carbon polyunsaturated fatty acids, usually from arachidonic acid (AA), and include prostaglandins, prostacyclins, thromboxanes, leukotrienes and other oxygenated derivatives, constitute the best-studied group of mammalian oxylipins.

1.1.1. The role and occurrence of prostaglandins

Prostaglandins are common signalling molecules in vertebrates. They regulate many physiologically important processes, such as body temperature and sleep-wake mechanisms, smooth muscle contraction/relaxation, Na⁺ excretion and fertility, as well as various pathological events, including inflammation, pain, fever and cancer. In addition, the occurrence of prostaglandins has been reported in several marine invertebrate species, e.g. crustaceans, cnidarians, molluscs, annelids, platyhelminthes and chordates, where they are mostly associated with reproduction, ion transport, immunity and defence reactions (Rowley et al., 2005). Several reports on the physiological roles of prostaglandins in marine and terrestrial invertebrates have been reviewed by Rowley et al., 2005; Stanley 2000; Stanley et al., 2009; Stanley and Kim 2011.

Prostaglandins have also been found in red and brown algae, and in traces in some fungi, but they have not been detected in flowering plants, mosses or prokaryotes (reviewed by Andreou et al., 2009; Brodhun and Feussner, 2011).

Interestingly, the prostaglandins formed in invertebrates and lower animals appear to fall into two categories, namely the classical prostaglandins (PGD₂, PGE₂ and PGF_{2α}), found in both invertebrates and vertebrates, and the novel prostaglandins, found only in invertebrates (Rowley et al., 2005). Marine invertebrates provide a rich source of classical and structurally unique oxylipins, including prostaglandins (reviewed by Gerwick et al., 1993). Therefore, the study of the pathways of oxylipin metabolism in marine invertebrates might reveal different or unique biosynthetic pathways (Gerwick, et al., 1993).

1.1.1.1. Prostaglandins in corals

The major fatty acid in the Caribbean coral *Plexaura homomalla* was found to be AA (Light, 1973). Furthermore, this coral is known to be the richest natural source of prostaglandins and their derivatives (especially PGA_2 and PGE_2 esters), containing up to 3-5% of its dry weight (Light and Samuelsson, 1972, Schneider et al., 1977). Interestingly, depending on the collection location, *P. homomalla* may contain prostaglandins in 15R- or 15S configuration (Schneider et al., 1977).

The Arctic soft coral *Gersemia fruticosa* is especially rich in AA (more than 60% of total fatty acids) and it contains the natural prostaglandins PGD_2 , PGE_2 and $\text{PGF}_{2\alpha}$, and 15-keto $\text{PGF}_{2\alpha}$ (Varvas et al., 1993; Varvas et al., 1999). The content of prostaglandins in the Arctic coral is several orders of magnitude lower than that of *P. homomalla* (0.008% of dry weight), but *G. fruticosa* has a significant ability to convert exogenous AA into optically active prostaglandins *in vitro* (Varvas et al., 1993; Varvas et al., 1999). It would seem that the high amounts of prostaglandins produced by the coral offers a chemical defence and acts as a deterrent to predatory fish (Gerhart, 1984).

Corals are also rich sources of unique eicosanoids. In the tropical Pacific soft coral *Clavularia viridis*, different novel prostaglandin-like compounds, such as clavulones, chloro-, bromo- and iodo-vulones, have been identified. The Hawaiian octocoral *Telesto riisei* contains unique prostaglandins: punaglandins. Additionally, methyl 11-acetoxy- $\text{PGF}_{2\alpha}$ and 11 α ,18-diacetoxy- $\text{PGF}_{2\alpha}$ have been identified in the Red Sea soft coral *Lobophyton depressum* (reviewed by Gerwick, et al., 1993). Several of these products have received a lot of attention due to their potential anti-tumour activity (Gerwick et al., 1993; Rowley et al., 2005). The exact function of these compounds in corals is unclear, but it is proposed that they provide defence against predatory fish and microbial attack (Rowley et al., 2005).

1.1.1.2. Prostaglandins in molluscs

In the Mediterranean ophistobranch mollusc *Tethis fimbria*, high levels of prostaglandin 1,15-lactones of both the E and F series have been discovered. The exact role of these compounds has not been determined, but it has been proposed that the novel prostaglandins play a role in reproduction, defensive behaviour and smooth muscle contraction (Marzo et al., 1991).

1.1.1.3. Prostaglandins in arthropods

In insects

Although the classical prostaglandins (PGE_2 , $\text{PGF}_{2\alpha}$ and PGD_2) and C_{20} polyunsaturated fatty acids have not been identified in insects, it is believed that

eicosanoids influence a range of actions in insect physiology, such as reproduction, ion transport, protein trafficking, immune system and defence reactions (reviewed by Stanley, 2000; Stanley and Miller, 2006; Stanley and Kim, 2011).

In 2011 Shrestha and colleagues cloned the first insect prostaglandin receptor in the beet army-worm *Spodoptera exigua*, which they named Se-hc-PGGPCR1, and they proved its role in insect reproduction. The receptor is a G protein-coupled receptor (GPCR) similar to mammalian prostaglandin receptors and it interacts with PGE₂ (Shrestha et al., 2011).

In crustaceans

Differently from terrestrial insects, crustaceans living in aquatic environments contain high proportions of C₂₀ polyunsaturated fatty acids, which are precursors in the biosynthesis of prostaglandins and other eicosanoids (Hanson et al., 1985; Stanley 2000).

Several studies on the effects of prostaglandins in crustacean reproduction have been published, and they demonstrate the presence of a prostaglandin biosynthetic system in the ovaries of different crustaceans. Classical prostaglandins have been detected in the ovaries of the shrimp *Penaeus monodon* (Wimuttisuk, et al., 2013), in the kuruma prawn *Marsupenaeus japonicus* (Tahara and Yano, 2004) and in the crab *Ozietelphusa senex senex* (Reddy et al., 2004), as well as in the previtellogenic ovary of the prawn *Macrobrachium rosenbergii* (Sagi et al., 1995). It has been demonstrated that prostaglandins (primarily PGE₂ and PGF_{2α}) play a vital role in reproductive development in female crustaceans, especially in the discharging of hatching factors, vitellogenesis and ovarian development (reviewed by Nagaraju 2011).

Additionally, PGE₂, thromboxane B₂ (TXB₂) and 6-keto-PGF_{1α} have been reported in the blood cells of the shore crab *Carcinus maenas*, although the physiological role of these eicosanoids is unknown (Hampson et al., 1992). PGE₂ has also been identified in the secretory products of the parasitic copepod crustacean *Lepeophtheirus salmonis*, in which it is thought to aid in parasite evasion by suppressing host immune responses (Fast et al., 2004).

1.1.1.4. Prostaglandins in fungi

Although some fungi (ascomycetes and basidiomycetes) contain high levels of C₁₈ polyunsaturated fatty acids, only traces of C₂₀ polyunsaturated fatty acids have been found in these organisms. It has been proposed that, in the case of pathogenic fungi, the host-derived AA may be used for prostaglandin synthesis (Andreou et al., 2009). So far it has been demonstrated that the fungal pathogens *Cryptococcus neoformans* and *Candida albicans* produce low amounts of PGE₂ from exogenously supplied AA (Erb-Downward and Huffnagle, 2007; Erb-Downward and Noverr, 2007). The enzymes responsible for prostaglandin biosynthesis have not been identified in fungi; therefore it is

proposed that the existence of a prostaglandin biosynthesis pathway in fungi is very unlikely and these compounds are formed by non-specific lipid peroxidation reaction (Brodhun and Feussner, 2011).

1.1.1.5. Prostaglandins in algae

Eukaryotic algae (red, green and brown algae, and diatoms) produce several polyunsaturated fatty acids, including AA (Guschina and Harwood, 2006), and they are also rich sources of different kinds of oxylipins (reviewed by Andreou et al., 2009). However, so far prostaglandins have only been identified in brown and red algae.

Prostaglandins in brown algae

Brown algae contain both C₁₈ and C₂₀ polyunsaturated fatty acids. Copper-induced stress in the *Laminaria digitata* led to a biosynthesis of a number of complex oxylipins, including prostaglandins: PGE₁, PGD₁, 15-keto-PGF_{2α}, PGE₂, 15-keto-PGE₂, PGA₂, PGB₂ and PGJ₂. These compounds are thought to trigger protective mechanisms in brown algae (Ritter et al., 2008; Andreou et al., 2009).

Prostaglandins in red algae

Marine red algae are rich sources of C₂₀ polyunsaturated fatty acids. The principal polyunsaturated fatty acid in most red algae is eicosapentaenoic acid (20:5n-3), whereas in several species of the genus *Gracilaria* a very high content of AA has been reported, varying from 46-62% of total fatty acids, depending on the season (Khotimchenko, 2005).

The occurrence of prostaglandins has been reported in different *Gracilaria* species: *G. lichenoides* (Gregson et al., 1979), *G. austramaritima* (Imbs et al., 2012), *G. chilensis* (Rempt et al., 2012) and *G. vermiculophylla* (Sajiki et al., 1998; Nylund et al., 2011), also known as *G. verrucosa* and *G. asiatica* (Skriptsova and Choi, 2009).

The main prostaglandins identified in red algae are PGE₂, PGA₂, PGF_{2α} and 15-keto PGE₂ (Gregson et al., 1979; Sajiki et al., 1998; Dang et al., 2010; Imbs et al., 2012). The content of PGE₂, which is the main prostaglandin found in red algae, is highest in the *G. vermiculophylla*, where it is up to 286 µg/g of dry weight, while in other *Gracilaria* species it is not above 20 µg/g (Imbs et al., 2012). The levels of prostaglandins in algae depend strongly on harvesting locations and cultivation conditions, as well as the season (Imbs et al., 2001; Imbs et al., 2012).

It has been proven that prostaglandins are important for the innate immunity and chemical defence system of the red algae (Nylund et al., 2011; Rempt et al., 2012). Additionally, prostaglandins, together with other oxylipins in red algae, are thought to be involved in ion transport and osmotic regulation (Gerwick et al., 1999).

In 1994 Noguchi et al. reported a food poisoning case resulting in one death, due to the ingestion of the edible red alga *Gracilaria verrucosa* (= *G. vermiculophylla*). This was thought to be caused by increased amounts of prostaglandins (especially PGE₂) and the further COX reaction that additionally increased the concentration of prostaglandins. This type of poisoning is very unusual and a better understanding of the prostaglandin biosynthesis pathways in red algae might prevent these situations in the future.

1.2. Enzymes responsible for the prostaglandin biosynthesis

In mammals, the biosynthesis of prostaglandins occurs through multiple enzymatically regulated reactions (Figure 1). The process begins with the release of AA from membrane phospholipids by the hydrolytic action of phospholipase A₂. The released AA is further metabolised into unstable endoperoxide intermediate PGH₂ by the actions of PG endoperoxide synthase, also called COX. All vertebrates have two COX isozymes, COX-1 and COX-2, which are differently regulated. The PGH₂ intermediate is further converted to different prostanoids by specific PGH₂ isomerases and reductases (Smith et al., 2011).

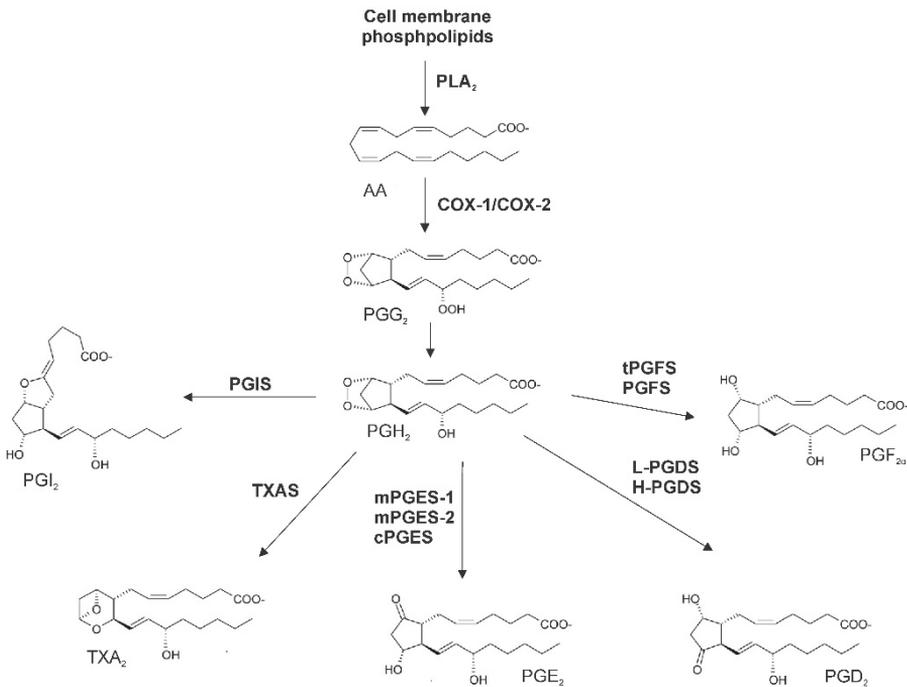


Figure 1. The prostaglandin synthesis pathway.

1.2.1. Phospholipases A₂

Phospholipases A₂ (PLA₂s) are comprised of distinct sets of enzymes with different localizations (Kudo and Murakami, 2002). They catalyse the hydrolysis of the sn-2 position of glycerol-phospholipids to yield fatty acids and lysophospholipids. Among them, the cytosolic PLA₂ (cPLA₂), secretory PLA₂ (sPLA₂) and Ca²⁺-independent PLA₂ (iPLA₂) families have been associated with eicosanoid production (Kudo and Murakami, 2002; Murakami et al., 2011). In

the cPLA₂ family, the cytosolic Ca²⁺-dependent cPLA₂α (85 kDa) plays a major role in the initiation of AA metabolism from cell membrane phospholipids (Ghosh et al., 2006). The enzymes in the iPLA₂ family work as phospholipases or lipases, with iPLA₂-β (88-90 kDa) being one of the best-studied members (Cedars et al., 2009). The sPLA₂ family (14-19 kDa) is involved in a number of biological processes, including eicosanoid generation, inflammation and host defence against bacterial infection (Murakami et al., 2011).

1.2.2. Cyclooxygenases

Cyclooxygenases (COXs), also known as prostaglandin H synthases or prostaglandin endoperoxide synthases (EC 1.14.99.1), catalyse two sequential enzymatic reactions: the bis-oxygenation of AA, leading to the production of PGG₂ (cyclooxygenase reaction), and the reduction of 15-hydroperoxide of PGG₂, leading to the formation of PGH₂ (hydroperoxidase reaction) (Figure 1; Smith et al., 2000).

All vertebrates investigated have two COX genes: one encoding the constitutive COX-1 and the other encoding the inducible COX-2. These two enzymes share 60-65% amino-acid identity. The orthologs of both COX-1 and COX-2 are very similar, sharing 70-95% and 70-90% amino acid sequence identity, respectively (Chandrasekharan and Simmons, 2004).

COX-1 is constitutively expressed and required for homeostatic functions, and is therefore called “the housekeeping” enzyme. COX-2 performs the dominant role in prostaglandin synthesis during pathological processes, such as inflammation, pain, fever and tumorigenesis, and is typically expressed at very low concentrations (Rouzer and Marnett, 2009).

1.2.2.1. Structure of the mammalian COXs

For convenience sake, the numbering of the amino acid residues in the description of the COX sequences and the protein structures is based on the sequence numbering in the ovine COX-1.

COXs are glycosylated, membrane-bound proteins found in the luminal surface of the endoplasmic reticulum (ER) and in the inner and outer membranes of the nuclear envelope. While COX-1 is more enriched in the ER than in the perinuclear envelope, COX-2 can be found mostly in the perinuclear envelope (Morita et al., 1995). Recent results suggest that COX-2 is also present in the Golgi apparatus (Smith et al., 2011).

Mature COX-1 and COX-2 have molecular masses of 67-72 kDa (Garavito and DeWitt, 1999). Although the enzymes are sequence homodimers, comprised of tightly associated monomers, they function as pre-existent conformational heterodimers, which consist of an allosteric (E_{allo}) and a catalytic monomer (E_{cat}) (Yuan et al., 2009; Dong et al., 2013). The

communication between the subunits (cross-talk) occurs through the dimer interface (Sidhu et al., 2010).

Each monomer of COX consists of an N- and a C-terminus, and three structural domains (Figure 2): an N-terminal epidermal growth factor (EGF)-like domain, a membrane-binding domain (MBD) and a globular C-terminal catalytic domain containing cyclooxygenase- and peroxidase-active sites (Picot et al, 1994; Garavito and Dewitt, 1999).

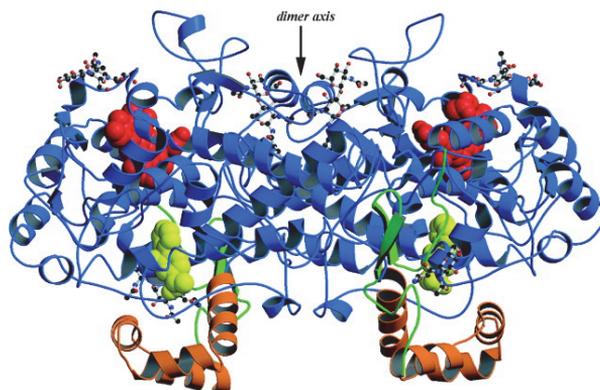


Figure 2. A crystal structure of the ovine COX-1 dimer (PDB entry 1CQE). The heme (red) and the binding site of flurbiprofen (yellow) are shown. The EGF, MBD and catalytic domains are coloured green, orange and blue, respectively. The N-linked sugars are shown in ball-and-stick rendering (Picot et al., 1994; Garavito et al., 1999, Garavito et al., 2002).

N-terminus

COX-1 and COX-2 isoforms have N-terminal cleavable signal peptides for their translocation into the lumen of the ER (Kulmacz et al., 2003). The signal peptide for the COX-1 is 22-26 amino acids long, and the signal peptide for the COX-2 is 17 amino acids in length and less hydrophobic. *In vitro* translation experiments suggest that the COX-1 translation rate is higher than that of COX-2, and this might be caused by the differences in the signal peptides (Simmons et al., 2004).

Right after the signal peptide of COX-1, there are eight amino acids which are not found in COX-2 (Simmons et al., 2004). This segment is believed to play a regulatory role in the cross-talk between the monomers comprising a COX dimer (Smith et al., 2011).

There are variants of COX-1 in which the intron-1 has been retained. In this case, the signal peptide is not cleaved, and it alters the biological properties of the enzyme (Simmons et al., 2004; Chandrasekharan et al., 2002).

EGF-like domain

The dimers of COX-1 and COX-2 are associated through hydrophobic interactions, hydrogen bonding and salt bridges between the dimerization regions in each monomer (Simmons et al., 2004). The EGF-like domain begins at the N-terminus of a mature COX enzyme, consists of approximately 50 amino acids and forms a part of the COX dimer interface (Simmons et al., 2004; Smith et al., 2000; Smith et al., 2011). It contains three intra-domain disulfide bonds that are absolutely conserved in both COX isoforms. There is one additional disulfide bond which links Cys37 and Cys159, and connects the EGF domain with the catalytic domain (Kulmacz et al., 2003). The EGF domain is thought to serve as a structural building block that initiates or maintains protein-protein interactions, and it also plays a role in protein-membrane interaction by fixing the MBD in position (Picot and Garavito, 1994).

Membrane-binding domain

Initially, the MBD of the COX enzymes was thought to be composed of four consecutive amphipathic α -helices: A, B, C and D. Three of the four helices (A, B and C) are located practically in the same plane, but the last helix (D) angles “upward” into the catalytic domain (Picot et al., 1994). Recent studies indicate that helix D is primarily a structural component of the cyclooxygenase (cox) active site (MirAfzali et al., 2006). The hydrophobic and aromatic amino acid residues in helices A, B and C form a hydrophobic surface that will interact with only one face of the lipid bilayer, connecting the cox channel to the phospholipid source of the fatty acid substrate (Picot and Garavito, 1994; Kulmacz et al., 2003). COX-1 and COX-2 are therefore monotopic membrane proteins with the ability to associate directly with the liposomes (Smith et al., 2000; MirAfzali et al., 2006). The highest sequence difference between COX isozymes occurs in the MBD, where there is 38% amino acid identity (Smith et al., 2011). The differences in these sequences indicate that the two COX isoforms associate differently with the membranes, which may affect their catalytic properties and biological activities (MirAfzali et al., 2006).

Catalytic domain

The catalytic domain is the largest part of the COX-1 and COX-2 proteins. It encompasses about 460 residues and consists of two functionally distinct regions, the cyclooxygenase (cox) and peroxidase active site (pox) areas (Picot et al., 1994; Smith et al., 2011). The catalytic domain has a globular structure and it is comprised of large and small intertwined lobes, which are mainly composed of conserved alpha-helical structures (Figure 3). The larger lobe has a V-shaped structure formed by the helices H5 and H6, with the helix H2 located just between them. The three core helices are surrounded by four helices (H3, H10, H18 and H19) to complete the large loop. The smaller lobe consists of six helices (H1, H8, H12, H14, H15 and H16), which form a bundle with their axes roughly parallel. The lobes are connected by six polypeptide chains, including helix H17 (Picot et al., 1994).

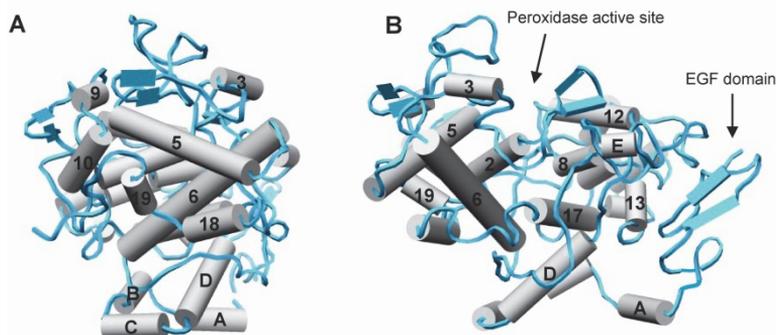


Figure 3. An alpha-helical structure of the ovine COX-1 monomer (PDB entry 1PRH). **A:** front view, and **B:** side view (Picot et al., 1994).

As mentioned before, the COX monomers are conformational heterodimers that consist of catalytic (E_{cat}) and allosteric monomers (E_{allo}) (Yuan et al., 2009). The cox-active site in E_{cat} has a catalytic function which is modulated by the nature of the ligand occupying the cox site of the partner, allosteric monomer (E_{allo}). Peroxidase activity also seems to appear only in the catalytic monomer of the enzyme (Dong et al., 2011; Smith et al., 2011).

The allosteric regulators that bind to the cox-active site of E_{allo} can be different fatty acids, even those that are not substrates for COX enzymes or different COX inhibitors, which are discussed in more detail below (Yuan et al., 2009; Dong et al., 2011; Smith et al., 2011).

The pre-existent conformational heterodimeric structure of COXs provides evidence that, initially, the active sites in the E_{cat} and E_{allo} monomers have slightly different structures. Additionally, ligand binding in the cox active site in these monomers induces even more subtle and reversible structural changes (Dong et al., 2013).

Cox-active site

The cox active site is a narrow hydrophobic dead-end channel, with a size of $8 \times 25 \text{ \AA}$. It extends from the MBD through helices A, B and C to the centre of the COX monomer (Picot et al., 1994). Both substrate and non-substrate fatty acids and different competitive COX inhibitors bind within the cox site. Each fatty acid and each inhibitor binds into one of the two preferred cox channels, in the E_{cat} or E_{allo} subunit (Smith et al., 2011; Dong et al., 2011). According to the amino acid sequence, this is the most highly conserved region of COXs; there is only one conservative amino acid residue substitution in the upper part of the channel, which is different in the two isoforms: Ile523 is a Val in COX-2 (Kulmacz et al., 2003; Simmons et al., 2004). The smaller Val generates a “side pocket” which allows bigger compounds to bind and inhibit this enzyme and is the key factor in the development of COX-2 selective inhibitors (Vane et al., 1998).

Tyr385 is located in the upper part of the channel, at the end of helix H8, and is surrounded by a ring of six residues in proximity to the heme iron (10 Å). Ser530 lies just below Tyr385. Tyr355, Arg120 and Glu524 are located towards the mouth of the cox channel, near the MBD. The last two residues are the only charged residues in the otherwise hydrophobic channel and may form a salt-bridge (Picot et al., 1994).

In the catalytic monomer of COX, Arg120, Tyr355 and Tyr385 are all important for substrate binding and catalysis and are well conserved in the cox active site (Thuresson et al., 2001, Garavito and Dewitt, 1999). However, in the allosteric monomer, some of these residues have different functions. In E_{allo} Arg120 is important for time-dependent inhibition by flurbiprofen and naproxen. Tyr385 in an allosteric monomer partners with Ser530 in allosteric regulation by non-substrate fatty acids (Dong et al., 2013). Ser530 in a catalytic monomer plays an important role in the efficiency of the enzyme; it is proposed that it participates in the formation of a Tyr385 radical and/or helps to locate the radical into the appropriate position (Schneider et al., 2002; Dong et al., 2013). Ser530 in the E_{cat} can be acetylated by aspirin and it therefore plays an important role in the inhibition of COXs (Dong et al., 2013).

Pox active site

The pox active site is located in a shallow cleft between the large and small lobes of the catalytic domain (Figure 3B). It is defined by helices H2, H5, H8 and H11/12. The helices H8 and H11/12 form one side of the cleft, while the helix H2 forms the opposite side. The floor of the cleft is formed by the helix H5 and the cleft is closed by two loop structures composed of the residues 210-222 and 394-408 (Picot et al., 1994; Gupta et al., 2004). The heme-binding site in the cleft is located diametrically opposite the MBD (Picot et al., 1994). Heme plays an important role in peroxidase activity, in the catalytic monomer of COX (E_{cat}), and the maximal COX activity occurs with a stoichiometry of only one heme per dimer (Kulmacz and Lands, 1984; Dong et al., 2011). The coordination of the heme occurs via an iron-histidine bond involving His388, which lies adjacent to helix H8. His207 and Gln203, which lie against the heme face, approximately 5 Å of the heme iron, are also important in peroxidase reaction (Picot et al., 1994; Gupta et al., 2004). There is remarkably low conservation in the side-chain structures between the two COX isoforms near the pox site, especially in the helix H11/12 and the structure of the pox site is considerably more stable in COX-1 than in COX-2 (Kulmacz et al., 2003).

C-terminus

The C-terminal region of COX-2 contains an 18-19 amino acid insert (depending on the species) which is missing in COX-1 (Kulmacz et al., 2003). These residues, with at least nine or ten residues upstream, are part of the instability motif that is associated with protein degradation and/or trafficking (Smith et al., 2011). Asn594, which is post-translationally glycosylated and

located inside this region, is also involved in COX-2 degradation (Mbonye et al., 2006; Smith et al., 2011).

Both COX isoforms contain C-terminal modified versions of KDEL-like sequences (STEL in most isoforms) that target COXs to the ER and the associated nuclear envelope. This sequence is thought to be a “weak” ER-retention signal and might provide for COX enzymes to traffic to the Golgi apparatus (Raykhel et al., 2007; Smith et al., 2000; Smith et al., 2011).

Glycosylation

Glycosylation is important for the correct folding of the COX enzymes. COXs are glycosylated on asparagine (Asn) residues in all mammals. COX-1 is glycosylated at three positions, while COX-2 may be glycosylated at up to four. In COX-1, potential sites for N-linked glycosylation are conserved at positions 68, 104, 144 and 410. The consensus site at 104 is absent in COX-2, but the enzyme has two additional N-glycosylation sites at positions 579 and 591. The actual N-glycosylation occurs at 68, 144 and 410 (and at 579 in COX-2) (Otto et al., 1993; Kulmacz et al., 2003).

1.2.2.2. Mechanism of the COX reaction

The cyclooxygenase and peroxidase reactions take place in the catalytic monomer of the COX. First, the heme group of the COX is oxidized to an oxyferryl porphyrin radical cation. The heme radical then oxidises Tyr385, which leads to the tyrosyl radical formation, which abstracts the 13 pro-*S* hydrogen of the AA-initiating cyclooxygenase reaction, which leads to the formation of an arachidonyl radical, which further reacts with O₂ and undergoes a complex intramolecular rearrangement to produce PGG₂. After that, the 15-hydroperoxyl group of PGG₂ is reduced to the corresponding alcohol of PGH₂ (peroxidase reaction) (Rouzer and Marnett, 2009; Dong et al., 2013).

1.2.2.3. COX substrate specificity

The most common substrates for COX enzymes are C₁₈ and C₂₀ polyunsaturated fatty acids, which contain at least three methylene-interrupted *cis*-double bonds (Smith and Marnett, 1991). AA (20:4 ω6) is the best substrate for both COX isoforms (Laneuville et al., 1995; Vecchio et al., 2010). Among C₂₀ fatty acids, dihomo-γ-linolenic acid (DGLA, 20:3 ω6) is also a good substrate for the COX enzymes, but eicosapentaenoic acid (EPA 20:5 ω3) is a poor substrate. The products are comprised of different prostaglandins: PGG₁ from DGLA, PGG₂ from AA and PGG₃ from EPA. 18-carbon fatty acids are oxidized to the monohydroxy products. All of these fatty acids are better substrates for COX-2 than for COX-1 (Laneuville et al., 1995).

The COX enzymes function as conformational heterodimers, and fatty acids that have not heretofore been associated with eicosanoid metabolism, including palmitic (16:0), stearic (18:0) and oleic (18:1 ω 9) acids, bind to the allosteric monomer and stimulate the oxygenation of AA in the catalytic monomer of COX-1 and/or COX-2. The efficiency of oxygenation is determined by the nature of the fatty acid bound to the allosteric monomer; therefore the exact efficiency in which the COX enzymes oxidise different fatty acids *in vivo* depends on the components available in the fatty acid pool (Yuan et al., 2009).

COX-2 is able to oxygenate various AA derivatives, such as the endocannabinoids - 2-arachidonoyl glycerol (2-AG), arachidonoyl ethanolamide (AEA) and N-arachidonoyl glycine (NAGly) - into corresponding endoperoxides and hydroxy acids (Vecchio and Malkowski, 2011). The best substrate among them is 2-AG, which is oxygenated as effectively as AA (Kozak et al., 2000). The ability of COX-2 to metabolize endocannabinoid substrates suggests that this isoform may be involved in the endogenous endocannabinoid signalling system (Vecchio and Malkowski, 2011).

There are three important structural differences that explain the different substrate preferences between the two COX isoforms. First, there are Val523, Arg513 and Val434 in COX-2, as opposed to Ile523, His513 and Ile434 in COX-1. The smaller side chains in COX-2 result in an \sim 25% increase in the volume of the cox channel (Rouzer et al., 2011; Vecchio et al., 2012). Second, the mobility of the Leu531 side chain in COX-2, which is located at the opening of the cox channel and above the residue Arg120, increases the volume at the mouth of the cox channel and permits the binding of endocannabinoid substrates (Vecchio et al., 2010). The third difference is the requirement of an ionic interaction between the carboxylate group of AA and the side chain of Arg120 for high affinity binding of substrate to COX-1 (but not in the case of COX-2). This suggests that hydrophobic interactions that appear between the ω -end of substrates and cox active site residues are essential in COX-2 catalysed oxygenation (Vecchio et al., 2012).

1.2.2.4. The inhibition of COXs

First, the COX inhibitors were separated into two categories: time dependent (which involve slow tight-binding inhibition) and time-independent (which display rapid, reversible inhibition) (Selinsky et al., 2001). The discovery of the pre-existent conformational heterodimeric nature of COX enzymes added previously unappreciated complexities to inhibitor and fatty acid interactions with COXs (Zou et al., 2012). It is now clear that some inhibitors function through E_{allo} , some through E_{cat} and some through both COX monomers (Smith et al., 2011). Additionally, non-substrate fatty acids also influence the inhibition process (Zou et al., 2012).

Aspirin and related nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX enzymes and thereby shut off the synthesis of all prostanoids, are

the most commonly used drugs worldwide (Grosser et al., 2006). Traditional NSAIDs inhibit both COX-1 and COX-2. COX-2 specific inhibitors, often referred to as coxibs, have greater selectivity toward COX-2 (Smith et al., 2011), and were designed to minimize gastrointestinal complications of traditional NSAIDs in their long-term use (Grosser et al., 2006). COX-2-selective NSAIDs, however, have been shown to cause increased risk of cardiovascular complications compared to non-selective NSAIDs (Grosser et al., 2010). COX-2 catalyses the synthesis of vascular PGI₂, which is an essential biologic vasodilator and also an inhibitor of platelet aggregation. The cardiovascular toxicity of COX-2 inhibitors is thought to be caused by an imbalance of prothrombotic thromboxane A₂ (TXA₂), produced by COX-1, and decreased production of antithrombotic PGI₂ (Mukherjee and Topol, 2003). The research for an effective and safe analgesic and anti-inflammatory drugs continues. Developing a new drug that targets the terminal enzymes responsible for prostanoid biosynthesis or individual prostanoid receptors might be the solution to the problem.

1.2.2.5. The structure of COX genes

Mammalian COX enzymes are encoded by distinct genes that map to different chromosomes. The human COX-1 gene maps to chromosome 9 (9q32-q33.3). The gene is ~22 kb in length with 11 exons and, according to the polyadenylation site used in the 3'-untranslated region (UTR), is transcribed as 2.8- or 5.2-kb mRNA variants. The gene for COX-1 represents a housekeeping gene and lacks a TATA box (Kraemer et al., 1992). There are two selective promoter factor 1 (Sp1) cis-regulatory elements in the human COX-1 promoter, which regulate transcription of the enzyme (Smith et al., 2000).

The COX-2 gene is very compact, maps to chromosome 1 (1q25.2-q25.3), is about 8 kb in length with 10 exons, and is transcribed as 2.8- and 4.6-kb mRNA variants. The promoter region of the COX-2 gene is characteristic of the immediate early gene and consists of a TATA box, as well as various transcription elements, such as a nuclear factor for interleukin-6 (NF-IL-6), activating protein-2 (AP-2), selective promoter factor 1 (Sp1), a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a cAMP response element (CRE) and an E-box. The 3'-UTR of the COX-2 mRNA is significantly longer (~1.5 kb) than that of the COX-1 and contains 22 copies of the AUUUA motif, which has been associated with RNA instability (Appleby et al., 1994; Murakami and Kudo, 2004).

1.2.2.6. COX in invertebrates

COX is an old gene represented in all vertebrates but it seems to have been lost in plants and some lower animals in the course of evolution. To date, non-

vertebrate COXs have been cloned and characterized from the soft corals *G. fruticosa* and *P. homomalla* (Koljak et al., 2001; Valmsen et al., 2001; Valmsen et al., 2004). Both corals have two COX isoforms which share about 80-93% of deduced amino acid sequence identity. Although there is relatively high homology (~50% amino acid identity) in the primary structure between coral and mammalian enzymes, there are significant structural differences in the region of the pox active site, in the MBD and in the location of the consensus glycosylation sequences (Koljak et al., 2001; Valmsen et al., 2001). Moreover one COX isoform from the coral *P. homomalla* has the unique ability to synthesize 15*R*-prostaglandins, which is caused by an amino acid interchange Val349Ile and four other residue substitutions in helices H5 and H6 (Valmsen et al., 2007).

COX has also been cloned and identified in the shrimp *P. monodon*, although the enzyme activity has not been examined (Wimuttisuk et al., 2013). There is bioinformatic evidence available of a possible COX pathway in different invertebrates. Using genome database analysis, COX genes have been reported in the primitive chordates *Ciona savignyi* and *Ciona intestinalis* (Järving et al., 2004), and in the crustaceans *Daphnia pulex*, *Homarus americanus* and *Petrolisthes cinctipes* (Heckmann et al., 2008).

All full length crustacean COXs share 45-50% amino acid identity with human COXs and contain catalytically important residues in their cox-active sites: Arg120, Tyr385, Ser530 (*D. pulex* has Ala in that position) and Val349. The proximal heme ligand His388 and the residues Gln203 and His207 are also conserved in the pox active centres of these enzymes. However, there are differences in the glycosylation pattern, as well as in the N-terminal and C-terminal domains. All of the known COX sequences from crustaceans lack the C-terminal KDEL-type ER retention/retrieval signal.

However, homologues of mammalian COX genes have not been identified in completely sequenced insect genomes of *Drosophila* sp., *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, *Tribolium castaneum*, *Danaus plexippus*, *Nasonia vitripennis* or others.

1.2.3. Prostaglandin synthases

After PGH₂ is formed by the action of COXs, it is converted into various prostanoids by specific PGH₂ isomerases and reductases (Figure 1). Two types of prostaglandin D synthases (PGDS) are responsible for PGD₂ synthesis; they are called lipocalyn-type PGDS and hematopoietic PGDS (Urade and Eguchi, 2002). In addition, two types of prostaglandin F synthases (PGFS) are responsible for the synthesis of PGF_{2α}. These are PGFS, which belongs to the aldo-keto-reductase (AKR) family (Watanabe 2002), and tPGFS, which belongs to the thioredoxine (Trx)-like enzyme family (Moriuchi et al., 2008). By the action of AKR-type PGFS, PGF_{2α} can also be synthesized from PGE₂ and 9α,11β-PGF₂ is synthesized from PGD₂ (Watanabe 2002). tPGFS also catalyses

the reduction of prostamide H₂ to prostamide F_{2α} (Moriuchi et al., 2008). In addition, there are three types of PGE synthases, which are described more precisely below.

1.2.3.1. Prostaglandin E synthases

In mammals, prostaglandin E synthase (PGES, EC 5.3.99.3), which isomerizes COX-derived PGH₂ specifically to PGE₂, occurs in three structurally and biologically distinct forms: cytosolic PGES (cPGES) and two membrane-bound PGES enzymes, designated as mPGES-1 and mPGES-2 (Kudo and Murakami, 2005).

cPGES

In 2000, a cytosolic form of PGES (23 kDa protein) was purified from lipopolysaccharide (LPS) treated rat brain. The enzyme requires glutathione (GSH) for its activity, is ubiquitously and constitutively expressed in different cells, and is therefore responsible for the production of the PGE₂ needed for the homeostatic functions. However, in rat brain, LPS treatment increases its amount several-fold. cPGES was initially called p23, a co-chaperone of heat shock protein 90 (Hsp90) (Tanioka et al., 2000; Kudo and Murakami, 2005), because the activation of cPGES requires its binding to Hsp90 and phosphorylation by casein kinase 2 (Murakami and Kudo, 2004). It has been shown that cPGES converts COX-1, but not COX-2-derived PGH₂ to PGE₂ (Murakami et al., 2002). Although cPGES is GSH-dependent, the amino acid sequence identity between cPGES and cytosolic glutathione S-transferases (GSTs) is only about 20%. In the N-terminus, cPGES has a conserved Tyr9 residue, which is also represented in many other cytosolic GSTs, in which it acts as a GSH acceptor. Mutation of Tyr9 abrogates the catalytic activity of cPGES (Garavito et al., 2002; Murakami and Kudo, 2004). Therefore, this residue is also thought to be involved with GSH binding in cPGESs (Salinas and Wong, 1999; Murakami and Kudo, 2004). cPGES is remarkably conserved, and the amino acid sequence identity across different vertebrate species is about 95%. The gene encoding for cPGES is localized to chromosome 12q13.13 and consists of 8 exons (Murakami et al., 2002).

cPGES is functionally coupled with COX-1, and thus their physiological functions *in vivo* overlap. COX-1-derived PGE₂ plays a role in gastrointestinal protection, reproduction and several neuronal functions which influence the central nervous system. cPGES knock-out mice are perinatal-lethal, with poor lung development, delayed skin maturation and growth retardation (Nakatani et al., 2007; Hara et al., 2010).

mPGES-1

mPGES-1, the first PGES identified, is a GSH-dependent protein which belongs to the MAPEG (**m**embrane-associated **p**roteins involved in **e**icosanoid and **G**SH

metabolism) family (Jakobsson et al., 1999). mPGES-1 (16 kDa protein) shows remarkable similarity to other MAPEG superfamily proteins, such as microsomal glutathione S-transferase-1 (MGST-1), MGST-2, MGST-3, 5-lipoxygenase-activating protein (FLAP) and leukotriene C4 synthase (LTC4S) (Murakami and Kudo, 2004; Hara et al., 2010). The mutation of Arg110 in mPGES-1, which is strongly conserved in all MAPEG proteins, alters its catalytic activity (Murakami et al., 2000). mPGES-1 converts COX-2-derived PGH₂ to PGE₂ and equivalently to COX-2, is induced by proinflammatory stimuli (delayed response) and is down-regulated by anti-inflammatory glucocorticoids. The crystallographic structure of mPGES-1 (3.5 Å), determined in 2008 (Jegerschöld et al., 2008) and more accurately (1.2 Å) in 2013 (Sjögren et al., 2013), confirmed the trimeric structure of the protein and similarity to MGST-1, the 5-lipoxygenase-activating protein and LTC4S. The gene for human mPGES-1 maps to chromosome 9q34.3 and consists of three exons (Forsberg et al., 2000).

mPGES-1 is expressed in various cell systems in which COX-2-derived PGE₂ is needed, such as inflammation, fever, pain, reproduction, tissue repair, bone metabolism and cancer. In addition, mPGES-1 is constitutively expressed in the kidney, and is therefore physiologically important in the urogenital tract (Thorén et al., 2003; Kudo and Muarakami, 2005). mPGES-1-deficient mice show reduced inflammatory reactions, decreased pain, febrile response and suppression of tumorigenesis (Hara et al., 2010).

mPGES-1 inhibitors

The long-term use of selective COX-2 inhibitors is associated with the increased risk of cardiovascular complications. The inhibitors that target the specific prostaglandin synthases/isomerases and reductases are thought to decrease these undesirable effects.

mPGES-1 acts as a target for the treatment of various inflammatory diseases and also for the treatment of cancer. Several inhibitors have been identified and developed to target mPGES-1. These compounds are distributed into three categories (Chang and Meuillet, 2011):

- Endogenous lipid, fatty acids and PGH₂ analogues
- Known anti-inflammatory drugs and/or inhibitors of leukotrienes biosynthesis
- Natural compounds

Several aspects complicate the development of appropriate mPGES-1 selective inhibitors, and selectivity is one of the biggest problem.

mPGES-1 belongs to the MAPEG family of proteins and, therefore, inhibitors that target the mPGES-1 will probably affect several other members of this family. Secondly, amino acid sequence differences between mPGES-1 proteins in humans, mice and rats complicate research. Finally, the enzyme has a very hydrophobic active site which probably exists in open and closed

conformations, which complicates modelling and drug-design. All those aspects make the development and discovery of selective inhibitors for mPGES-1 very difficult (Chang and Meuillet, 2011). Although a lot of research has been conducted to develop mPGES-1 inhibitors, no clinical trials have yet been completed (Smith et al., 2011; Korotkova and Jakobsson, 2014). So far, only one novel mPGES-1 inhibitor, GRC 27864, has successfully completed pre-clinical and phase one enabling studies.

mPGES-2

The second membrane-associated form of PGES was initially purified from a microsomal fraction of bovine heart (Watanabe et al., 1999), and later cDNAs-encoding human and monkey homologues were identified (Tanikawa et al., 2002). The enzyme is a 41 kDa protein which is structurally different from mPGES-1 (Hara et al., 2010). mPGES-2 is initially synthesized as a Golgi-membrane-associated protein, and the further proteolytic removal of the N-terminal hydrophobic domain (87 amino acid residues) leads to the formation of a mature cytosolic enzyme. The full-length and N-terminal-truncated mPGES-2 have identical catalytic properties (Tanikawa et al., 2002). The N-terminal hydrophobic domain is followed by a glutaredoxin/thioredoxin homology region, in which the consensus of the thioredoxin homology sequence of Cys110-x-x-Cys113 is presented (Tanikawa et al., 2002; Hara et al., 2010). Unlike cPGES and mPGES-1, the enzyme does not require GSH for its catalytic activity, although the activity is increased two- to four-fold in the presence of SH-reducing reagents. The recombinant mPGES-2 is activated by dihydrolipoic acid, dithiothreitol, GSH and β -mercaptoethanol, in order of decreasing effectiveness (Tanikawa et al., 2002; Watanabe et al., 2003).

mPGES-2 is mainly expressed in the brain, heart, skeletal muscle, kidney and liver (Tanikawa et al., 2002). It has been shown that mPGES-2 can be coupled with both COX-1 and COX-2. In most tissues, the protein is constitutively expressed, and its concentration is not increased during inflammation or tissue damage. However, a significant increase in mPGES-2 expression level has been detected in human colorectal cancer (Murakami et al., 2003, Kudo and Murakami, 2005, Hara et al., 2010).

The crystal structure of recombinant monkey mPGES-2 (Figure 4) was reported in 2005, in complex with indomethacin. It was revealed that mPGES-2 forms a homodimer and is attached to the lipid bilayer by anchoring the N-terminal section (Yamada et al., 2005).

The dimer of mPGES-2 is comprised of two hydrophobic pockets which are connected to form a V-shaped structure. According to the structure, the SH group of Cys110 is probably the catalytic site of the protein. The substrate, PGH₂, fits well into the V-shaped pockets and its endoperoxide moiety can easily interact with the SH group of Cys110. In spite of the low amino acid sequence identity, the crystal structure of mPGES-2 is quite similar to that of GSH-dependent hematopoietic PGD synthase (H-PGDS) and several classes of GSTs (Yamada et al., 2005, Smith et al., 2011).

Interestingly, mPGES-2 knock-out mice showed no differences in their phenotype and in PGE₂ levels in several tissues (including liver, kidney, heart and brain). The PGE₂ concentration did not change even in LPS-stimulated macrophages. Therefore, it is proposed that mPGES-2 is not involved in PGE₂ synthesis under the physiological and pathological conditions examined so far (Hara et al., 2010).

However, it has been demonstrated that the mPGES-2 knock-out mice treated with streptozotocin to induce type-1 diabetes exhibited severe lethality and liver toxicity a few days after the treatment. The authors proposed that mPGES-2 deficiency robustly increased streptozotocin-induced liver toxicity via the increase in glucose transporter 2 (GLUT2) dependent uptake of streptozotocin (Sun et al., 2014).

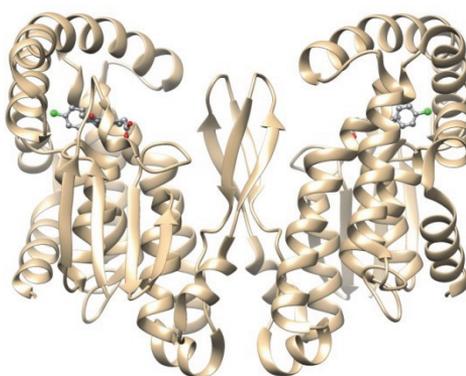


Figure 4. A crystal structure of *Macaca fascicularis* mPGES-2 in complex with indomethacin. Residues 1-99, which are part of the N-terminal section and disorder section, are not shown. The structure (PDB:1Z9H) was constructed with Chimera 1.10.

In 2013 Takusagawa reported that macaque mPGES-2 exists in two forms, as heme-free and heme-bound enzymes, that the heme-free enzyme catalyses the formation of PGE₂ from PGH₂, and that the heme-bound mPGES-2 is a GSH-dependent protein which catalyses PGH₂ degradation to 12(S)-hydroxy-5,8,10(Z,E,E)-heptadecatrienoic acid (12-HHT) and malondialdehyde (MDA). As the heme-free recombinant mPGES-2 converts to the heme-bound form if free heme is available, it was proposed that macaque mPGES-2 is a PGE₂ synthase *in vitro* but not *in vivo* (Takusagawa 2013).

The human mPGES-2 gene is located in 9q34.11, in proximity to the genes encoding for mPGES-1, COX-1 and lipocalin-type PGDS (Tanikawa et al., 2002). The gene of mPGES-2 is about 7.7 kb in length and consists of 7 exons.

1.2.3.2. PGES in invertebrates

Surprisingly, PGES-like sequences are quite common in arthropod genomes. While more than 30 predicted mPGES-2-like sequences have been identified in insects and other arthropod genomes so far, there is little information about the catalytic activity of corresponding proteins.

All three PGES sequences have been described only in the peaneid shrimp *P. monodon* (Wimuttisuk et al., 2013). Prostaglandin synthase activity was also observed in the shrimp ovary homogenates using *in vitro* assay, although no individual enzyme activity has been described (Wimuttisuk et al., 2013). Based on bioinformatic evidence, putative genes associated with eicosanoid biosynthesis have also been described in the water flea *D. pulex* (Heckmann et al., 2008). Those findings support the hypothesis that the prostaglandin synthesis pathway in aquatic crustaceans is similar to that of mammals.

The situation is more complicated among terrestrial insects, where mostly mPGES-2-type PGES is represented. Campbell et al. (2009) used *L. salmonis* putative mPGES-2 as a gene knock-down target gene. They cloned and sequenced a putative mPGES-2 from the sea louse. The authors were uncertain whether this enzyme is involved in PGE₂ synthesis or performs some other function. The temporal and tissue distribution of *L. salmonis* PGES-2 indicated that it is a ubiquitous and constitutively expressed gene, more like a detoxifying GST than a gene involved in PGE₂ synthesis (Campbell et al., 2009). *Drosophila melanogaster* has a mPGES-2 homologue protein called a suppressor of ref(2)P sterility (Su(P)) (Bichon et al., 2001). The enzyme has an N-terminal hydrophobic domain, which is membrane-associated, and a C-terminal soluble domain, with a GST-like structure characteristic to mPGES-2 proteins. Bichon et al. (2001) proved that in association with the ref(2)P (a protein that plays a critical role in mediating the autophagy of defective mitochondria (Pimenta de Castro et al., 2012)) the enzyme plays a role in male fly fertility, but the exact function of the enzyme remains unclear (Bichon et al., 2001).

1.2.3.3. Sigma-class glutathione transferase exhibiting prostaglandin E synthase activity

A sigma class GST exhibiting *in vitro* both PGE₂ synthase- and GSH transferase activity was recently described in the silkworm *B. mori*, and named bmGST1 (Yamamoto et al., 2013). Although COX has not been identified in completely sequenced insect genomes, it is possible that enzymes involved in the prostaglandin synthesis pathway differ from their mammalian counterparts in those organisms. At the same time, there is a possibility that *in vivo*, sigma-class GSTs have only GSH transferase activity. This hypothesis is also supported by the fact that polyunsaturated fatty acids, precursors in the biosynthesis of prostaglandins, are not found in terrestrial insects. The development of selective

and non-selective inhibitors of bmGST1 should help establish the physiological role of bmGST1 in the silkworm life cycle (Yamamoto et al., 2013).

1.2.4. COX-related proteins

Zamocky et al. (2008) aligned mammalian heme containing peroxidases, including COXs, and similar peroxidase domain-containing sequences from all kingdoms of life. This enzyme superfamily contained proteins with a variety of functions that could be grouped in seven subfamilies and they named this group of enzymes the peroxidase-cyclooxygenase superfamily. According to phylogenetic analysis and the ability to oxygenate fatty acids, α -dioxygenases (α -DOX) are functionally related to COX (Zamocky et al., 2008; Goulah et al., 2013). Linoleate diol synthases (LDS), which are fusion enzymes with N-terminal dioxygenase and C-terminal cytochrome P450 activity, were found in fungi. The dioxygenase domain in LDS shares structural similarities with both α -DOX and COX (Hoffmann et al., 2011).

α -dioxygenases

In 1998 a new protein, called pathogen-induced oxygenase (PIOX), was identified in tobacco leaves (Sanz et al., 1998); this enzyme was also named α -dioxygenase (α -DOX). α -DOXs are heme-containing enzymes which oxygenate 14-20 carbon fatty acids, containing up to three double bonds, into the corresponding 2*R*-hydroperoxides. They are mostly found in plants and fungi, where they are up-regulated during the host defence response against pathogen attack (Hamberg et al., 2005; Zhu et al., 2013). Despite the low sequence identity (~15%) with COXs, these enzymes share striking similarities in tertiary structure and catalytic features.

α -DOX is a monomeric protein which lacks the EGF domain but has a catalytic domain and a base domain analogous to MBD. The base domain is not structurally similar to MBD; it consists of eight α -helices, of which three are amphipathic. However, compared to COXs, it is located in a similar position with respect to the catalytic domain and serves as an entry for the substrate into the active site channel (Goulah et al., 2013; Zhu et al., 2013). Within the catalytic domains of *Arabidopsis thaliana* and *Oriza sativa* α -DOX and COX-2, 21 of the 22 α -helices are conserved. Helices H2 and H8, which contain the distal and proximal histidine residues and form the binding cleft for heme, as well as helices H6 and H17, which form part of the active site channel, are all conserved (Goulah et al., 2013; Zhu et al., 2013).

The reaction catalysed by α -DOX is unique, in that oxygenation takes place at the α -carbon, not at the bis-allylic position, as in COX proteins (Zhu et al., 2013). In contrast to COX, α -DOX binds the fatty acid with the carboxylate group bond deep within the active site channel, near Tyr386 (which is analogous to Tyr385 in COXs). The removal of the pro-*R* hydrogen from the α -carbon is mediated by the Tyr386 radical. The latter is produced via the

oxidation of the heme with hydrogen peroxide (H₂O₂). α -DOXs also contain an additional Ca²⁺-binding loop, which is important for Tyr386 radical formation. Because of the restricted access to the distal face of the heme caused by the two extended inserts on the surface of the enzyme, α -DOXs have limited peroxidase activity (Goulah et al., 2013; Zhu et al., 2013).

Linoleate diol synthases

7,8- and 5,8-LDS, which share 22-24% amino acid identity with COXs, are heme-containing fatty acid dioxygenases found in fungi. The enzymes are fusion proteins of N-terminal domains with 8*R*-DOX activities and C-terminal cytochrome P450 domains with diol synthase activities. Therefore, the N-terminal ends of 5,8- and 7,8-LDS are structurally related to DOX and COX. 7,8- and 5,8-LDS oxidise 18:2n-6 sequentially to (8*R*)-hydroperoxylinoleic acid (8*R*-HPODE) and to (5*S*,8*R*)- or (7*S*,8*S*)-dihydroxyoctadecadienoic acids (DiHODE). Just like COXs and α -DOXs, the enzymes form a tyrosyl radical for hydrogen abstraction during catalysis and have conserved distal His residue, important for heme coordination (Garscha and Oliw, 2007; Hoffmann et al., 2011).

Other COX-related proteins

Although the myeloperoxidases (MPO) share only 20% identity with COXs and catalyse different reactions, the overall topology of their catalytic domains is strikingly similar. The MPO has conserved helices H2, H5, H6, H8 and H12, involved with the heme binding. The structure homology extends over the entire catalytic domain, but myeloperoxidases lack the MBD and EGF domain (Picot et al., 1994).

Peroxinectins (Pxt) are heme-containing proteins with peroxidase domains and an integrin-binding motif (KGD: Lys-Gly-Asp). They are found among various arthropods and nematodes, in which they regulate physiologically important processes, such as fertility and immunity (Vizzini et al., 2013). Furthermore, the Pxt from *D. melanogaster* is thought to serve as a putative COX (Tootle and Spradling, 2008). Still, the further characterization of these enzymes from insect species may explain their exact function.

The phylogenetic analysis made by Zamocky et al. (2008) revealed that, although COX, MPO and Pxt enzymes had a common ancestor, and they have retained some common structural elements, they segregated very early in evolution to perform different functions.

2. AIMS OF THE STUDY

The general goal of the current study was to unravel the pathway and to characterize the enzymes involved in the prostaglandin biosynthesis in lower marine organisms, more specifically in red algae and amphipod crustaceans.

The specific aims of the current study were:

- To establish the metabolic pathway of prostaglandin biosynthesis in red algae. To clone, express and characterize the enzymes involved.
- To search for and characterize the enzymes responsible for the biosynthesis and further metabolism of prostaglandin endoperoxides in amphipod crustaceans. To clone, express and characterize amphipod COXs. To identify the enzymes involved in the synthesis of PGE₂. More specifically, to clone and characterize mPGES-2, as the most widespread PGES in arthropods.

3. METHODS

The methods used in this work, are described in detail in the original publications I-III added to the thesis. The methods are:

gDNA isolation

RNA isolation

Reverse transcriptase polymerase chain reaction (RT-PCR) cloning

Protein expression and purification

Western blotting

Protein characterization

Bioinformatic analysis

Database linking:

The algal COX sequences described in this work have been submitted to the GenBank database under the accession no **JN565603** (*Gracilaria vermiculophylla* PGHS) and no **JN565604** (*Coccotylus truncatus* PGHS).

The amphipod COX sequences studied in this work have been deposited in the GenBank database under accession no **GQ180795** (*Caprella* sp. COX) and no **GQ180796** (*Gammarus* sp. COX).

The mRNA sequences of amphipod mPGES-2 have been submitted to the GenBank database under the accession no **KC832830** (*Caprella* sp.) and no **KC832831** (*Gammarus* sp.) and the protein sequences under accession no **AGO64144** (*Caprella* sp.) and no **AGO64145** (*Gammarus* sp.).

4. RESULTS

4.1. Structural and catalytic characterization of COXs in red algae (I)

COXs from red algae were cloned by the RT-PCR strategy. The His-tagged proteins were expressed in *E. coli* BL21(DE3)RP cells and purified with nickel nitrilotriacetic acid column and gel filtration chromatography. The cyclooxygenase activity of the purified enzymes was determined by a fibre optic oxygen monitor and the peroxidase activity was evaluated spectrophotometrically. The products formed from [¹⁴C]AA were identified by reversed-phase high-pressure liquid chromatography (RP-HPLC). The heme content of the purified recombinant COX was determined by a pyridine-hemochromogen assay. The oligomeric state of the algal COX was examined with blue native polyacrylamide gel electrophoresis and gel filtration chromatography. The main results of the study are as follows:

- COXs from the red algae *G. vermiculophylla* and *C. truncatus* share only about 20% of amino acid identity with their mammalian counterparts.
- The algal COXs lack structural elements identified in all known mammalian COXs, such as the N-terminal signal peptide, the EGF-like domain, the helix B of the MBD and the KDEL(STEL)-type ER retention/retrieval signal.
- The predicted tertiary structure for the *G. vermiculophylla* COX monomer (65 kDa) suggests a conservation of overall structural architecture with mammalian COXs. However, the algal COX has several differences in the catalytically important amino acid residues.
- Tyr385 (catalytically essential) and Val349 (important for oxygenation stereo control at C-15) are conserved in *G. vermiculophylla*. However Arg120, Tyr355 and Ser530, important for substrate binding and coordination, are substituted by Leu120, Ser355 and Thr530, respectively. These substitutions are presumably responsible for the ability of the enzyme to oxidize amide and ester derivatives of fatty acids. Additionally, the Ser530Thr substitution explains the insensitivity of the algal COX to aspirin, and the Arg120Leu substitution is likely responsible for the inactivity of the reversible inhibitors flurbiprofen and indomethacin.
- The cox active site geometry in the *G. vermiculophylla* enzyme is more similar to that of mammalian COX-2 than COX-1. The algal enzyme has a “side pocket” in the cox active site, which is formed by the sterically smaller amino acid residues Leu434, Asp513 and Ala523, compared to Ile434, His513 and Ile523 in COX-1. In that position, mammalian COX-2 also has the sterically smaller amino acids Val434, Arg513 and Val523.

- The heme ligand His388 and residues Gln203 and His207, important for peroxidase activity, are conserved in both algal COXs and the peroxidase activity of the *G. vermiculophylla* enzyme is comparable to the ovine COX.
- Despite the presence of N-glycosylation sites, *G. vermiculophylla* COX does not need N-glycosylation for proper folding. The enzyme expressed in the prokaryotic expression system was fully functional and catalysed the conversion of AA to prostaglandin endoperoxides.
- *G. vermiculophylla* COX was detected as oligomeric (evidently tetrameric) ferric heme protein.
- The preferred substrate for *G. vermiculophylla* COX is AA, and the average specific activity of purified algal COX is 120–130 units/mg of protein, which is 3-5 times higher than previously reported values for human COXs. DGLA was oxygenated with lower efficiency and EPA was a poor substrate. The algal COX oxidizes fatty acid amide and the ester derivatives AEA and 2-AG to the corresponding prostaglandin products, at rates of 28% and 14%, respectively, compared to AA.
- The COXs in red algae are encoded by intron-free genes; in the *G. vermiculophylla*, the gene is about 2.2 kb long and in the *C. truncatus* about 1.9 kb.

4.2. Prostaglandin synthesis in crustacean arthropods (II, III)

4.2.1. Genetic and biochemical characterization of crustacean COXs (II)

COX-related cDNA was cloned from the amphipod crustaceans *Gammarus* sp. and *Caprella* sp. by the RT-PCR strategy. To determine whether amphipod COX proteins are glycosylated, expression in COS-7 cells was performed in the presence or absence of the N-glycosylation inhibitor tunicamycin. The recombinant amphipod enzymes were expressed in COS-7 cells and baculoviral Sf9 cells. The activity of the enzymes was measured using microsomal fractions of the COS-7 and Sf9 cells, containing recombinant amphipod COXs, and the products were identified by RP-HPLC. To determine the subcellular localization of amphipod COX proteins, an indirect immunofluorescence microscopy analysis was used.

- COXs from the crustacean amphipods *Gammarus* sp. and *Caprella* sp. are 68 kDa enzymes, which share about 40-46% amino acid sequence identity with the human COX-1 and COX-2.
- The major differences between amphipod and mammalian COXs are in their N- and C-terminal regions. The N-terminal signal sequence in amphipod enzymes is not cleaved, and the C-terminus is shorter than that of mammalian isoforms and lacks the KDEL(STEL)-type ER retention/retrieval signal.
- Amphipod COX proteins contain key residues shown to be important for cyclooxygenase and peroxidase activities. In a cox active site, Arg120, Tyr355, Ser530 (important for substrate binding and coordination) and Val349 (oxygenation stereo control at C-15) are all conserved. In a pox active site, His388 (a heme proximal ligand), Gln203 and His207 (important for peroxidase activity) are conserved in both amphipod enzymes.
- The cellular localization of amphipod COX proteins is similar to that of the mammalian counterparts: they locate on the ER and nuclear envelope.
- Amphipod COX proteins are glycosylated, although they share only two of three consensus sites with mammalian counterparts. Gammarid and caprellid COXs have the third potential N-glycosylation site at Asn213, and the gammarid COX has an additional consensus site at Asn439.
- The recombinant COX proteins, expressed in both baculoviral Sf9 cells and in COS-7 cells, were fully functional and catalysed the same reactions as mammalian counterparts: the enzymes converted AA into prostaglandin endoperoxide, which degraded to a mixture of the stable end products PGF_{2α}, PGE₂ and PGD₂ in an aqueous medium.

- The non-selective inhibitor of mammalian COX indomethacin and the selective COX-2 inhibitor nimesulide were not able to inhibit amphipod COXs.
- The COX gene from *Gammarus* sp. is ~9.6 kb long and has thirteen exons; the COX gene from *Caprella* sp. is ~6.4 kb long and has eleven exons.

4.2.2. Membrane-associated prostaglandin E synthase-2 from crustacean arthropods (III)

The cDNA sequences encoding mPGES-2 were cloned from the amphipod crustaceans *Gammarus* sp. and *Caprella* sp. using the RT-PCR strategy. The His-tagged proteins were expressed in *E. coli* BL21(DE3)RP cells and purified with nickel nitrilotriacetic acid column and continuous diafiltration. The activity of the purified enzymes was measured using a coupled enzyme assay in which the substrate for mPGES-2, PGH₂, was synthesized from [¹⁴C]AA, using highly active COX from the red alga *G. vermiculophylla*. The reaction products were identified by RP-HPLC. The heme content of the purified amphipod protein was measured spectrophotometrically. Titration of the purified mPGES-2 with heme solution was monitored using a spectrophotometer.

- The mPGES-2 proteins from *Gammarus* sp. and *Caprella* sp. have a molecular mass of about 50 kDa and they share 40-43% identity with the human mPGES-2. The highest (48-51%) amino acid sequence homology of amphipod mPGES-2s was found with PGES-2-like sequences of the prawn *P. monodon*, sea lice *Caligus rogercresseyi* and water flea *D. pulex*.
- Like mammalian mPGES-2, amphipod enzymes consist of N-terminal membrane-associated regions and cytoplasmic glutathione S-transferase (cGST)-like regions, which include thioredoxin-like domains, and C-terminal helical domains. The glutaredoxin/thioredoxin homology region includes the catalytically important Cys-Pro-Phe-Cys motif. The predicted GSH-binding motif is also conserved in amphipod proteins.
- Amphipod mPGES-2 has the longest N-terminal part compared to other known mPGES-2s, a long C-terminus and some insertions larger than two amino acids in the sequence.
- The predicted protein structure model of caprellid mPGES-2 shows remarkable similarity to the *Macaca fascicularis* mPGES-2 crystal structure. The superimposed structures share about 40% of identical residues. The root-mean-square deviation (RMSD) between 233 α -carbon atom pairs (79% of total) was 0.840 Å.
- N-terminally truncated amphipod mPGES-2 enzymes can be functionally expressed in the prokaryotic expression system.
- The amphipod mPGES-2 proteins have drastically lower heme-binding capacity than their mammalian counterparts, and therefore amphipod mPGES-2 may exist *in vivo* as heme-free proteins capable of isomerizing PGH₂ to PGE₂.
- Both, the crude cell extract containing the recombinant mPGES-2 and the purified recombinant mPGES-2 converted algal COX-derived PGH₂ specifically to PGE₂. The addition of heme to the assay mixture did not initiate the degradation of PGH₂ to 12-HHT and MDA.

- GSH increases both the stability and activity of amphipod mPGES-2, while a sodium salt of the well-known SH-group inhibitor p-hydroxymercuribenzoate completely inhibits the mPGES-2 activity.
- The mPGES-2 genes from caprellid and gammarid consist of seven exons and span about 4.1 kb and 4.3 kb of DNA, respectively.

DISCUSSION

The occurrence of prostaglandins and other oxylipins has been reported in several marine organisms, including red algae. However, the mechanism of prostaglandin biosynthesis in non-animal organisms has remained unknown for years. In recent decades, genes with a certain homology to animal COX genes have been identified in plants, fungi and bacteria. These genes encode for proteins that, in spite of some structural similarity with animal COX, differ in catalytic activities. For example, fatty acid dioxygenases, α -DOXs, show striking similarities compared to animal COXs, with 21 out of 22 α -helices, and catalytically important tyrosine is conserved, yet they catalyse the oxygenation of fatty acids to the corresponding 2*R*-hydroperoxides (Goulah et al., 2013).

The initial goal of the present study was cloning and the structural and biochemical characterization of the enzymes responsible for the prostaglandin synthesis in red alga *G. vermiculophylla*. Using degenerative primers and the RT-PCR cloning strategy, several COX-like sequences were identified. However, it was found that most of them belonged to the arthropods, more specifically to amphipod crustaceans, inhabiting the red algal community.

Although, prostaglandins have been shown to play certain regulatory roles in insects and other arthropods, acting on reproduction, the immune system and ion transport, knowledge of their biosynthetic pathways in arthropods is lacking. Moreover, homologs of vertebrate COX genes have not been identified in completely sequenced terrestrial insect genomes, and it was proposed that in arthropods the enzymes involved in prostaglandin biosynthesis differ from their mammalian counterparts. The peroxinectin (Pxt) from *D. melanogaster* is thought to serve as a putative COX (Tootle and Spradling, 2008) and peroxidases (POXs) from *S. exigua* are also believed to be associated with prostaglandin biosynthesis (Park et al., 2014). The only bioinformatic evidence of the possible COX pathway in arthropods is derived from crustaceans (Heckmann et al., 2008). Here, our second goal developed: to add some clarity regarding the biosynthetic pathways of prostaglandins in aquatic arthropods.

The first non-animal COX which we identified in the red alga *G. vermiculophylla* displays atypical structural features. The algal COX has no N-terminal signal peptide and lacks structural elements identified in all known animal COXs, such as the EGF-like domain and helix B in the MBD. The recombinant protein was identified as a tetrameric protein. With the loss of the EGF-domain, the three intra-domain disulfide bonds that are absolutely conserved in both COX isoforms are also lost. Therefore, it can be speculated that the subunits of red algal COX are differently associated compared to the mammalian enzymes, and the cross-talk between the monomers (if it occurs) has to be regulated in different ways. In addition, there are questions about the substrate uptake and the subcellular localization of the enzyme.

The vertebrate COX proteins are very similar to each other. There is 60-65% sequence identity between COX-1 and COX-2 from the same species and 85-90% identity among individual isoforms from different vertebrate species

(Smith et al., 2000). The algal COX shares only 20% amino acid sequence identity with its mammalian counterparts, but the comparison of its predicted tertiary structure with the x-ray crystal structure of ovine COX-1 suggests a high conservation of overall structural architecture. However, on the basis of modelling data, differences in the primary structure of algal COX cause significant conformational changes in the MBD and in the entry to the substrate binding channel. X-ray studies of algal COX, preferentially co-crystallized with substrate analogues, may answer these questions.

Our studies clearly show that the prostaglandin biosynthesis pathway, similar to that of mammals, is also used by red algae. It is noteworthy that the COX pathway is not only represented in the red alga *G. vermiculophylla* from the Sea of Japan, but a very similar COX sequence was identified in the red alga *C. truncatus* inhabiting the almost fresh water of Kassari Bay in the Baltic Sea.

Compared to the red algal enzymes, the COXs from crustacean arthropods are more similar to their mammalian counterparts. The major differences in primary structure between amphipod and mammalian COXs lie in the N- and C-termini of proteins. The N-terminal signal peptide is not cleaved in amphipod COXs, and they lack the C-terminal KDEL(STEL) ER retention/retrieval signal, yet the proteins are post-translationally processed and localize, similarly to mammalian enzymes, in the ER and nuclear envelope. All of the known crustacean COX sequences have remarkably short C-termini. Although it has been shown that amino acid substitutions or deletions at the C-terminus of COX-1 (but not COX-2, which has a longer C-terminus more tolerant to structural changes) led to a significant loss of catalytic activity (Guo and Kulmacz, 2000), the extremely short C-terminus of amphipod COXs seems not to disturb their catalytic activity.

Glycosylation has been shown to be important for the correct folding and activity of the COX proteins (Kulmacz et al., 2003). While N-linked glycosylation sites are strongly conserved in vertebrate COXs, the glycosylation pattern of COXs in lower marine organisms seems to be more heterogeneous (Figure 5). Site-directed mutagenesis studies with mammalian COXs have established that glycosylation at Asn410 is essential for mammalian COX activity (Otto et al., 1993). This consensus site is not conserved in invertebrate and algal enzymes. In coral (*P. homomalla* and *G. fruticosa*) COXs, there are three to six N-glycosylation consensus sequences, and the crustacean amphipod *Gammarus* sp. and *Caprella* sp. COXs have four and three N-glycosylation sites, respectively. Only one of the conserved N-glycosylation sites in vertebrate COX isozymes, at Asn144, is present in invertebrate COXs; in crustacean enzymes, the positions at Asn68 are also conserved, but the locations of all the other sites in invertebrate enzymes are different. In spite of essential differences in their N-glycosylation patterns, the COXs from corals and amphipods have to be glycosylated to be functional. The red algal *C. truncatus* and *C. vermiculophylla* COXs have three and two potential N-glycosylation sites, respectively, but none of them is conserved with animal COXs. However they share one consensus site, Asn193, with coral enzymes. More interestingly, the

algal COX is unique, because it does not need N-glycosylation, and it can be expressed in prokaryotic systems as a fully functional enzyme. Furthermore, the recombinant algal COX was not directed to the N-glycosylation machinery when expressed in eukaryotic COS-7 cells. During the late phase of our research, Kanamoto et al. also reported the identification of a COX gene from the red alga *G. vermiculophylla* and expression of functional recombinant algal COX in *E. coli* cells (Kanamoto et al., 2011). They showed that recombinant *E. coli* cells produced PGF_{2α} in a medium supplemented with AA, and secreted the PGF_{2α} product.

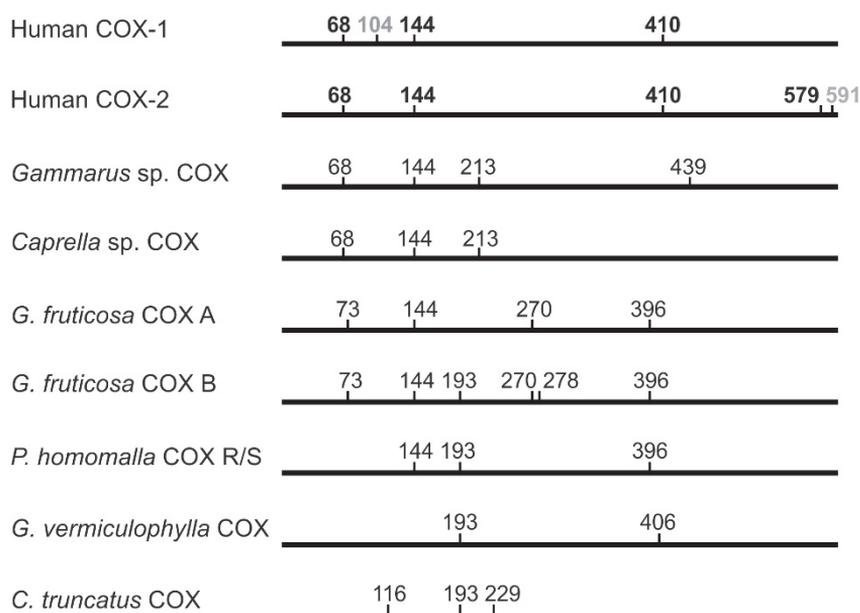


Figure 5. Consensus sequences for N-linked glycosylation of mammalian and lower marine organism COXs. In mammalian COXs, consensus sites which are actually glycosylated are shown in bold, and the other potential N-glycosylation sites are shown in grey. The figure is constructed using multiple sequence alignment of deduced amino acid sequences of human, amphipod, coral and algal COXs and it does not reflect the actual protein lengths in amino acids.

Non-specific NSAIDs inhibit the cyclooxygenase activities of both COX-1 and COX-2, while COX-2 specific inhibitors called coxibs are more potent towards COX-2. Aspirin causes covalent modification of mammalian COX through irreversible acetylation of highly conserved Ser530. The other NSAIDs inhibit enzymes by competing with AA at the cyclooxygenase active site (Simmons et al., 2004). Our studies showed that Ser530Thr substitution in the algal COX effectively rendered the enzyme insensitive to aspirin, while the product profile of the algal COX did not change, unlike the respective mutants of animal COX. Other non-specific NSAIDs and COX-2 selective nimesulide,

even at high inhibitor concentrations, only very weakly affect the algal enzyme activity. The Arg120Leu substitution is probably the reason for the insensitivity of algal COX toward NSAIDs containing carboxylic acid moieties, such as flurbiprofen and indomethacin. In amphipod COXs, Arg120 is conserved. Surprisingly, our studies showed that indomethacin did not inhibit the activity of the recombinant amphipod COX. There are several studies describing the inhibition of biological processes in insects with mammalian COX inhibitors indomethacin and aspirin. These discrepancies indicate how variable and complicated the biological processes in insects are and many of these are still waiting to be studied.

The phylogenetic analysis (Figure 6) revealed that while vertebrate COX sequences are strictly divided into COX-1 and COX-2 groups, the COX sequences of different classes of invertebrates form separate arms and show considerable divergence, even though essential catalytic residues are conserved. This confirms the hypothesis that vertebrate COXs share a common ancestor with their invertebrate counterparts and that the COX-1 and COX-2 segregated later in evolution, after the divergence of the Animal Kingdom into vertebrates and invertebrates (Järving et al., 2004). The red algal COX probably diverged even earlier, in the course of divergence of algal and animal lineages, evolved differently and preserved more primordial features characteristic of ancestral enzyme, while the animal COXs have acquired novel structural elements. α -DOXs presumably diverged during the separation of algal and plant lineages and have remained ancestral features.

While mammals, birds and amphibians have two COX isoforms, the constitutively expressed COX-1 and inducible COX-2, some types of fish have three COX genes (Ishikawa and Herschman, 2007). The situation in lower marine organisms is even more complicated. Only one COX sequence has been identified in both red algae and amphipod crustaceans investigated so far, as well as in the genome of the cladoceran crustacean *D. pulex* (Heckmann et al., 2008) and the malacostracan crustacean *P. monodon* (Wimuttisuk et al., 2013). Although two COX genes have been identified in corals and sea squirts, they emerged from gene duplication, independently of the vertebrate COX-1 and COX-2 divergence (Järving et al., 2004). It is clear that some lower animals lost the COX gene in the course of evolution. Crustaceans, in which many COX-like sequences have been found, are believed to represent the ancestral arthropods from which insects originated (Glennier et al., 2006). So far, direct homologs of mammalian COX genes have not been identified in the completely sequenced insect genomes *Drosophila* sp., *A. aegypti*, *A. gambiae*, *B. mori*, *T. castaneum* and others. However, using bioinformatics analysis, we identified the COX-like sequence in one insect, the human body louse *P. humanus corporis*. The body louse COX shares about 30% identity with mammalian COXs and the comparison of intron positions and phases, as well as exon sizes, of the *P. humanus* COX gene suggests a common evolutionary origin with vertebrate enzymes.

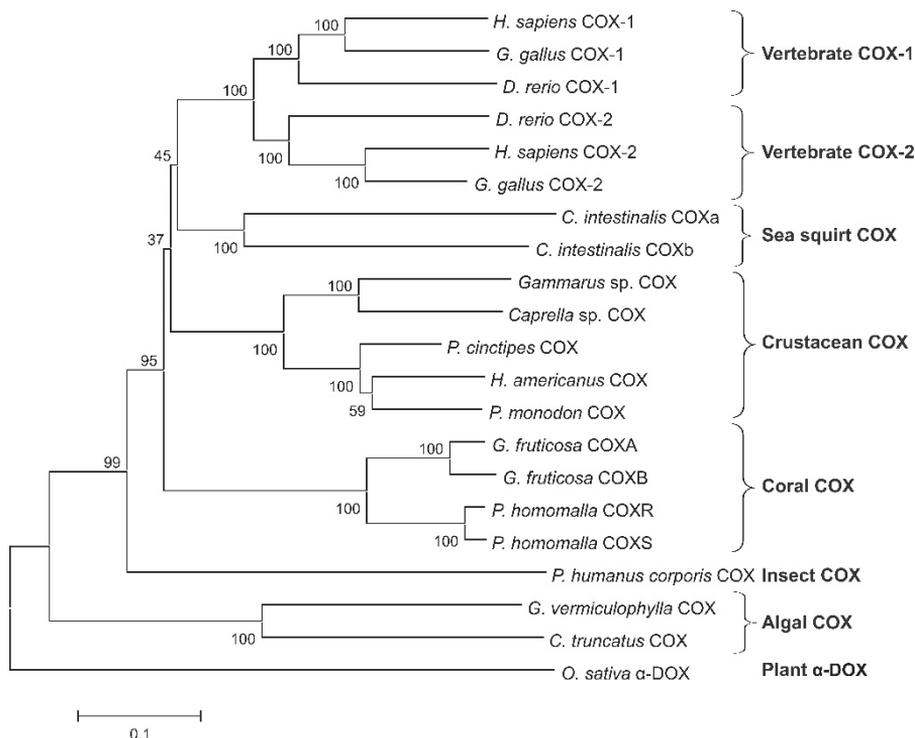


Figure 6. The phylogenetic tree of the α -DOX and COXs. The tree was constructed with MEGA 5.10 using amino acid sequences with the neighbour-joining method. The scale bar represents an estimate of the number of amino acid substitutions per site. Bootstrap values are indicated for each branch divergence.

In lower marine organisms, the knowledge of PGH₂ isomerases and reductases, the next enzymes in the prostaglandin biosynthesis pathway, is also lacking. mPGES-2-like sequences have been found in many arthropod genomes, including in insect genomes, where COX-like sequences are missing. Therefore, it was of interest to us to clone and characterize the first mPGES-2 proteins from arthropods. The phylogenetic analysis revealed that arthropod mPGES-2 sequences form clearly distinct clusters compared to vertebrate enzymes (Figure 8, in Article III), indicating their early segregation and independent development.

Takusagawa has reported that macaque mPGES-2 exists in two forms, as heme-free and heme-bound enzymes, that the heme-free enzyme catalyses the formation of PGE₂ from PGH₂, and that the heme-bound mPGES-2 is a GSH-dependent protein which catalyses PGH₂ degradation to 12-HHT and MDA. It was proposed that mammalian mPGES-2 is a GSH-dependent heme protein and that *in vitro* dithiothreitol dissociates the bound heme to produce active heme-free PGE₂ synthase (Takusagawa, 2013).

To avoid heme dissociation, we purified amphipod mPGES-2 enzymes in the absence of dithiothreitol and in the presence of GSH. Our results show that

mPGES-2 enzymes in aquatic arthropods have significantly lower heme-binding affinity than mammalian mPGES-2. Therefore, they may exist *in vivo* as heme-free proteins capable of catalysing the synthesis of PGE₂.

Since PGH₂ is extremely unstable in an aqueous solution, *in vitro*, the catalytic activity of amphipod mPGES-2 was studied in a “one-pot process”, in which PGH₂ was both generated and metabolised without isolating the intermediate. The substrate for mPGES-2, PGH₂, was synthesized from [¹⁴C]AA, using a highly active recombinant COX from the red alga *G. vermiculophylla*. Both amphipod mPGES-2 proteins converted the algal COX-derived PGH₂ specifically to PGE₂.

The mPGES-2 is the only enzyme, so far, that is known to change its catalytic activity in the presence or absence of the cofactor (Yamada and Takusagawa, 2007; Takusagawa 2013). In this way, the enzyme can carry out different physiological roles.

Our data demonstrate that amphipods use the prostaglandin synthesis pathway, where PGE₂ is formed from AA via successive reactions of COXs and mPGES-2. The exact role of mPGES-2-like enzymes in terrestrial insects and other invertebrates lacking COXs and their substrate fatty acids has yet to be determined.

In summary, the results of the current study clearly indicate that all organisms, animal or non-animal, use similar routes, i.e. COX pathways of prostaglandin synthesis. All COXs originate from a common ancestor and, despite significant structural differences established through evolution, they have maintained their catalytic specificity.

CONCLUSIONS

In this work, the enzymes responsible for prostaglandin biosynthesis in the marine organisms red algae and crustacean amphipods were studied. It was proved that the prostaglandin synthesis pathway in red algae and crustaceans are similar to that of mammals. It was also demonstrated that amphipods use the prostaglandin synthesis pathway where PGE₂ is formed from AA via successive reactions of COX and mPGES-2. The main conclusions of the thesis are as follows:

- COXs from red algae (*G. vermiculophylla* and *C. truncatus*) were cloned and characterized. The algal COXs share only about 20% amino acid identity with their animal counterparts, yet catalyse the conversion of AA into prostaglandin-endoperoxides, PGG₂ and PGH₂. The enzymes lack several structural elements identified in all known animal COXs, such as the EGF domain and helix B in MBD. The catalytically important Tyr385 and heme ligand His388 are conserved in the algal enzyme, but the amino acids important for substrate binding and coordination (Arg120, Tyr355 and Ser530) are not found at the appropriate positions. Algal COXs can be functionally expressed in *E. coli*. The preferred substrate for the algal COXs is AA but, similarly to animal COX-2, it is capable of metabolizing ester and amide derivatives of AA to corresponding prostaglandins. Algal COX is not inhibited by NSAIDs.
- COXs from marine crustacean amphipods (*Gammarus* sp. and *Caprella* sp.) were expressed and characterized. The amphipod proteins contain key residues important for cyclooxygenase and peroxidase activities, and catalyse the same reactions as their mammalian counterparts. The N-terminal signal sequence of the amphipod COX is not cleaved and the C-terminus is shorter than that of mammalian isoforms and lacks the KDEL(STEL)-type ER retention/retrieval signal, yet the amphipod enzymes are glycosylated and locate on the ER and nuclear envelope. The amphipod COX is not inhibited by NSAIDs.
- mPGES-2 from the same amphipods were examined. The amphipod mPGES-2 proteins share 40-43% amino acid identity with human mPGES-2, contain a conserved Cys110-x-x-Cys113 motif and have drastically lower heme-binding affinity than mammalian enzymes. The recombinant purified amphipod mPGES-2 enzymes specifically catalyse the isomerization of PGH₂ to PGE₂. The PGES activity is increased in the presence of GSH and inhibited with a sulfhydryl group inhibitor.

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ABSTRACT

Cyclooxygenases (COX) catalyse the first two steps in the biosynthesis of prostaglandins, well-known lipid mediators in vertebrates, which regulate many physiologically important processes and various pathological events. The occurrence and physiological roles of prostaglandins have been reported in several lower marine organisms. However, very little is known about the enzymes responsible for their biosynthesis. The first invertebrate COXs have been identified in the soft corals *Plexaura homomalla* and *Gersemia fruticosa*. COX proteins have never previously been identified in arthropods or in algae. Although COX-like sequences have been detected in different aquatic crustaceans, the homologs of vertebrate COX genes have not been identified in fully sequenced terrestrial insect genomes. Therefore, it is believed that in some arthropods the enzymes involved in prostaglandin biosynthesis may significantly differ from their mammalian counterparts.

The aim of the current study was to unravel the biosynthetic pathways of prostaglandins in lower marine organisms. Using the RT-PCR strategy, we cloned and characterized COXs in the algae *Gracilaria vermiculophylla* and *Coccolytus truncatus*, as well as in the amphipod crustaceans *Gammarus* sp. and *Caprella* sp. In addition, membrane-associated prostaglandin E synthase-2 (mPGES-2) enzymes in the same amphipods were cloned and described.

COXs from red algae share only about 20% of the amino acid sequence homology with their animal counterparts and reveal atypical structural features. They lack some structural elements identified in all known animal COXs, as well as several amino acid residues shown to be important for substrate binding and coordination. Differently from animal COXs, *G. vermiculophylla* COX expresses in *E. coli* as a fully functional enzyme that is not inhibited by non-steroidal anti-inflammatory drugs (NSAIDs). Similarly to animal COX-2, algal COX is capable of metabolising ester and amide derivatives of arachidonic acid to the corresponding prostaglandin products.

In marine amphipod COXs, the structural elements and key residues important for cyclooxygenase and peroxidase activities are conserved. However, there are remarkable differences in the C- and N-termini of the amphipod proteins. Both amphipod recombinant COXs expressed in COS-7 cells catalyse the conversion of the AA into prostaglandin endoperoxides, PGG₂ and PGH₂, and are not inhibited by NSAIDs.

The next enzymes in the prostaglandin biosynthesis pathway, the amphipod mPGES-2 proteins, share about 40% amino acid sequence identity with their mammalian counterparts. Interestingly, these enzymes have remarkably lower heme binding affinity compared to mammalian mPGES-2s, and most probably they exist *in vivo* as heme-free proteins capable of catalysing the synthesis of PGE₂. The sulfhydryl group inhibitor p-hydroxymercuri-benzoate totally inactivates amphipod mPGES-2 proteins, indicating the catalytical importance of the Cys110-x-x-Cys113 motif.

In conclusion, the first non-animal COX from marine red algae was characterized. We demonstrated that amphipods use the prostaglandin synthesis pathway, where PGE₂ is formed from arachidonic acid via successive reactions of COX and mPGES-2. It was shown that the enzymes involved in prostaglandin synthesis in lower marine organisms significantly differ from their mammalian counterparts, yet maintain their catalytic specificity. The current study of arachidonic acid metabolism in lower marine organisms provides new insights into the evolutionary, structural and catalytic aspects of enzymes responsible for prostaglandin biosynthesis.

KOKKUVÕTE

Loomsetes organismides katalüüsivad tsüklooksügenaasid (COX) kahte esimest etappi oluliste lipiidsete signaalmolekulide, prostaglandiinide sünteesis. Kuigi prostaglandiinide leidumist ning nende füsioloogilist rolli on kirjeldatud mitmes alamas mereorganismis, on vähe teada neid ühendeid sünteesivatest ensüümidest ja biosünteesi radadest. Esimesed selgrootute COX-d identifitseeriti korallidest *Plexaura homomalla* ja *Gersemia fruticosa*. Andmed COX-dest lüljalgsetes ja vetikates on siiani puudulikud. Kuigi COX-i sarnaseid järjestusi on leitud erinevatest meres elavatest vähilistest, pole täielikult järjestatud maismaa putukate genoomidest identifitseeritud selgroogsete COX geeni homoloogi. Seetõttu on arvatud, et mõnedes lüljalgsetes võivad prostaglandiinide sünteesis osalevad ensüümid oluliselt erineda imetajate vastavatest ensüümidest.

Antud töö eesmärgiks oli välja selgitada, milline prostaglandiinide biosünteesi rada on kasutusel alamates mereorganismides. Kasutades RT-PCR strateegiat, kioneeriti ja iseloomustati punavetikatest *Gracilaria vermiculophylla* ja *Coccotylus truncatus* ning kirpvähilistest *Gammarus* sp. ja *Caprella* sp. COX-d. Samuti kioneeriti ja kirjeldati samade kirpvähiliste membraanseoselised prostaglandiin E süntaas-2 (mPGES-2).

Punavetikate COX-d omavad vaid 20% aminohappelise järjestuse identsust selgroogsete COX-dega ning on ebatüüpilise struktuurse ülesehitusega. Neil puuduvad mõned struktuurselt olulised elemendid, mis on esindatud kõigis selgroogsete COX-des ning mitmed substraadi sidumiseks ja koordineerimiseks vajalikud aminohappejäägid. Erinevalt imetajate COX-dest, on prokarüootses süsteemis ekspresseeritud *G. vermiculophylla* COX katalüütiliselt aktiivne ning ei ole inhibeeritav mittesteroidsete põletikuvastaste ainete poolt. Sarnaselt selgroogsete COX-2-le, võib punavetika COX kasutada substraadina ka arahhidoonhappe ester- ja amiid-derivaate.

Uuritud kirpvähiliste COX-des on struktuurselt olulised elemendid ning tsüklooksügenaasi ja peroksüdaasi aktiivsuseks vajalikud aminohappejäägid konserveerunud. Märkimisväärseid erinevusi leidub aga valkude N- ja C-terminaalsetes otstes. Siiski on COS-7 rakkudes ekspresseeritud rekombinantsete kirpvähiliste COX-d aktiivsed ning katalüüsivad arahhidoonhappe konversiooni prostaglandiin-endoperoksiidideks, PGG₂ ja PGH₂. Ka need ensüümid ei ole mittesteroidsete põletikuvastaste ainete poolt inhibeeritavad.

Prostaglandiinide sünteesiraja järgmised ensüümid, kirpvähiliste mPGES-2-d omavad 40% aminohappelise järjestuse identsust vastavate imetajate ensüümidega. Huvitaval kombel omavad need ensüümid aga viimastega võrreldes märkimisväärselt madalat heemi sidumise afiinust ning eksisteerivad tõenäoliselt *in vivo* heemivabal kujul, olles võimelised katalüüsima PGE₂ sünteesi. Sulfüdrüülrühma inhibiitor p-hüdroksü-Hg-bensoaat inaktiveerib kirpvähiliste mPGES-2 täielikult, mis omakorda viitab ensüümis leiduva Cys110-x-x-Cys113 motiivi katalüütilisele tähtsusele.

Antud töös tõestati esmakordselt, et imetajatele sarnane prostaglandiinide sünteesi rada on esindatud ka mitteloomset päritolu organismides – identifitseeriti ja iseloomustati punavetika COX. Leiti, et mere kirpvähilistes toimub prostaglandiinide süntees arahhidoonhapest läbi COX-rajaga. Kuigi vastavad ensüümid alamates mereorganismides erinevad oluliselt imetajate ensüümidest, on nad siiski säilitanud oma katalüütilise spetsiifilisuse. Antud uurimistöö arahhidoonhappe metabolismist alamates mereorganismides annab täiendavat informatsiooni prostaglandiinide biosünteesirajas osalevate ensüümide evolutsiooniliste, struktuursete ja katalüütiliste aspektide osas.

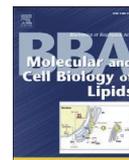
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Structural and catalytic insights into the algal prostaglandin H synthase reveal atypical features of the first non-animal cyclooxygenase[☆]

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ABSTRACT

Prostaglandin H synthases (PGHSs) have been identified in the majority of vertebrate and invertebrate animals, and most recently in the red alga *Gracilaria vermiculophylla*. Here we report on the cloning, expression and characterization of the algal PGHS, which shares only about 20% of the amino acid sequence identity with its animal counterparts, yet catalyzes the conversion of arachidonic acid into prostaglandin-endoperoxides, PGG₂ and PGH₂. The algal PGHS lacks structural elements identified in all known animal PGHSs, such as epidermal growth factor-like domain and helix B in the membrane binding domain. The key residues of animal PGHS, like catalytic Tyr-385 and heme liganding His-388 are conserved in the algal enzyme. However, the amino acid residues shown to be important for substrate binding and coordination, and the target residues for nonsteroidal anti-inflammatory drugs (Arg-120, Tyr-355, and Ser-530) are not found at the appropriate positions in the algal sequences. Differently from animal PGHSs the *G. vermiculophylla* PGHS easily expresses in *Escherichia coli* as a fully functional enzyme. The recombinant protein was identified as an oligomeric (evidently tetrameric) ferric heme protein. The preferred substrate for the algal PGHS is arachidonic acid with cyclooxygenase reaction rate remarkably higher than values reported for mammalian PGHS isoforms. Similarly to animal PGHS-2, the algal enzyme is capable of metabolizing ester and amide derivatives of arachidonic acid to corresponding prostaglandin products. Algal PGHS is not inhibited by non-steroidal anti-inflammatory drugs. A single copy of intron-free gene encoding for PGHS was identified in the red alga *G. vermiculophylla* and *Coccolytus truncatus* genomes.

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1. Introduction

Prostaglandins are formed by the action of prostaglandin H synthase (PGHS; EC 1.14.99.1), which catalyzes the committed step in the conversion of arachidonic acid (AA; 20:4 ω -6) to the prostaglandin-endoperoxides PGG₂ and PGH₂ [1,2]. The PGHS genes have been identified in all the vertebrates investigated, including mammals, birds, teleosts, and cartilaginous fish. In vertebrates, two PGHS genes and proteins exist that are differently regulated. PGHS-1 is expressed

constitutively in most tissues, whereas PGHS-2 is inducible in response to stimulation of cells with a very broad range of agonists [3].

The occurrence of prostaglandins and other oxylipins has also been reported in several marine organisms: in invertebrate species (reviewed by Gerwick [4] and Rowley [5]) and in eukaryotic algae (reviewed by Gerwick [6], Guschina [7] and Andreou [8]). While the prostaglandin-endoperoxide pathway of prostaglandin biosynthesis in non-vertebrate marine animals has been demonstrated in corals [9–11] and in crustaceans [12], the prostaglandin biosynthetic pathway in non-animal organisms has remained unknown for years. Recently, a PGHS gene was identified in the red alga *Gracilaria vermiculophylla* [13]. The PGHS gene cloned from the alga appears to be functional when expressed in a prokaryotic expression system. *Escherichia coli* cells produced PGF_{2 α} in a medium supplemented with substrate AA and the product PGF_{2 α} accumulated in culture medium.

Different from green plants, marine red algae are rich sources of C₂₀ polyunsaturated fatty acids, which are precursors in the biosynthesis of prostaglandins and other eicosanoids. The dominant polyunsaturated fatty acid in most red algae is eicosapentaenoic acid (20:5 ω -3), whereas in several species of the genus *Gracilaria* a very high content of AA has been reported (varying from 46 to 62% depending on the season) [14]. Occurrence of prostaglandins has been

Abbreviations: PGHS, prostaglandin H synthase; GvPGHS, *Gracilaria vermiculophylla* PGHS; CtPGHS, *Coccolytus truncatus* PGHS; huPGHS, human PGHS; AA, arachidonic acid; PG, prostaglandin; AEA, arachidonoyl ethanolamide; AG, arachidonoylglycerol; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; RACE, rapid amplification of cDNA ends; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TMPD, N,N,N',N'-tetramethylphenylenediamine; NSAID, non-steroidal anti-inflammatory drug; BN-PAGE, blue native polyacrylamide gel electrophoresis; RP-HPLC, reverse phase high-performance liquid chromatography; TLC, thin layer chromatography; LC-MS, liquid chromatography mass spectrometry; UTR, untranslated region; EGF, epidermal growth factor

[☆] Data deposition: The sequences reported in this paper have been deposited in the GenBank. *Gracilaria vermiculophylla* PGHS GenBank ID: JN565603 and *Coccolytus truncatus* PGHS GenBank ID: JN565604.

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reported only in the *Gracilaria* species: *G. lichenoides* [15], *G. asiatica* [16,17], *G. verrucosa* [18,19], *G. vermiculophylla* [20,21] and *G. chilensis* [21]. It has been shown that prostaglandins, hydroxylated fatty acids, and other AA derived oxylipins are part of the innate defense system of the red algae *G. vermiculophylla* and *G. chilensis* [20,21].

Both PGHS isoforms of animal origin are homodimers composed of tightly associated monomers with identical sequences, and dimerization is required for structural integrity and catalytic activity [22,23]. In PGHS dimer, each monomer has a physically distinct peroxidase and cyclooxygenase active site, but only a single high affinity heme binding site per dimer [24,25]. Recent studies have indicated that both PGHS isoforms function as conformational heterodimers during catalysis and inhibition [26,27]. PGHSs are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, and indomethacin [28,29]. PGHS-1 has a strong requirement for a free carboxyl group in the substrate, whereas PGHS-2 efficiently utilizes neutral derivatives (esters and amides) of AA as substrates [30,31].

In this work, we characterized the structure and function of *G. vermiculophylla* PGHS (GvPGHS). Biochemical and structural similarities, as well as differences in algal PGHS in comparison with mammalian PGHS are discussed.

2. Materials and methods

2.1. Materials

Samples of the red alga *G. vermiculophylla* (*Rhodophyta*; *Florideophyceae*; *Gracilariales*) were collected from the coast of Kanagawa Prefecture in Tokyo Bay. Red algal *Coccolytus truncatus* (*Rhodophyta*; *Florideophyceae*; *Gigartinales*) samples were collected on the coast of the Baltic Sea in Kassari Bay. The samples were transported and stored at -80°C .

The fatty acids, 2-arachidonoyl glycerol (2-AG), arachidonoyl ethanolamide (AEA), indomethacin and flurbiprofen were purchased from Cayman Chemical (Ann Arbor, MI). Anapoe X-100 was purchased from Anatrace, Inc. (Maumee, OH). The oligonucleotides used in this study were purchased from DNA Technology (Denmark). Restriction enzymes were purchased from MBI Fermentas. All other chemicals were obtained from Sigma-Aldrich.

2.2. RT-PCR-cloning

Total RNA from the algal samples was extracted as described previously [32]. mRNA was prepared from the total RNA by using mRNA purification kit (QIAGEN). The first strand cDNA was prepared using an oligo(dT)-adaptor primer [33]. Initial PCR fragments were obtained using degenerate primers based on the conserved regions of known PGHS sequences, as described previously [34]. 5'-RACE was accomplished using a 5'-RACE kit (Roche Diagnostics). The oligonucleotide sequences used in this study are given in Table S1 (Supplementary). All the PCR products were cloned into the pGEM-T Easy vector (Promega), amplified in *E. coli* DH5 α , and sequenced (LGC Genomics, Germany).

cDNA for the full-length GvPGHS was obtained by PCR, using the proofreading *Pfu* polymerase (Promega), and 1 μl of the first strand cDNA as a template. The primers MVF-forward and PV-reverse (Supplementary Table S1), with the *Nde*I site added at the 5' end of both primers to facilitate subcloning, were used. The digested and purified PCR products were cloned into the pET-11a vector. Four clones of GvPGHS constructs were full-length sequenced and expressed in *E. coli* cells. To facilitate protein purification, a hexahistidine (His_6) tag was added upstream of the start codon of GvPGHS by PCR. The His_6 -GvPGHS was cloned into the pET-11a vector, and the construct was sequenced and expressed in *E. coli* BL21(DE3)RP cells.

2.3. Amplification and cloning of genomic DNA

The genomic DNA was isolated from clean tips of *G. vermiculophylla* and *C. truncatus* thalli using the CTAB procedure, as described previously [12]. To obtain the genomic sequences of GvPGHS and CtPGHS, gene-specific primers were used (Supplementary Table S1). The PCR products were cloned and sequenced as described above. The genomic DNA sequences were compared with the cDNA sequences.

2.4. Bioinformatics

Tertiary structure modeling was conducted using a CPHModels 3.0 algorithm [35]. The model was built using 1DIY from <http://www.rcsb.org> as a template.

2.5. Bacterial expression and purification of His-tagged protein

Recombinant plasmid of His_6 -GvPGHS was transformed into *E. coli* BL21(DE3)RP cells. Cells containing His_6 -GvPGHS recombinant plasmids were grown at 37°C in an LB medium containing ampicillin to a density of 0.8–0.9 D at 600 nm. After chilling to 25°C , δ -aminolevulinic acid (a final concentration of 0.2 M), hemin ($5\ \mu\text{M}$), and isopropyl- β -D-thiogalactopyranoside (0.5 mM) were added, and the culture was continued for 18 h at 25°C . The cells were harvested by centrifugation and stored at -70°C until protein purification.

All purification steps were performed at 4°C . Harvested cells (6–7 g from 1 l culture medium) were re-suspended in 50 ml of 20 mM Tris-HCl, pH 8.0 containing 1 mM phenylmethylsulfonyl fluoride. Lysozyme was added to 0.2 mg/ml, followed by incubation for 30 min on ice. Then the suspension was sonicated (Bandelin Sonopuls) with twelve cycles of 10 s burst/3 min cooling on ice. The membrane fraction was collected by centrifugation for 1 h at 100,000 \times g. The pellet was re-suspended in solubilization Buffer A (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), sodium deoxycholate was added dropwise to the final concentration of 1%, and gentle stirring continued for 2 h. Solubilized lysate was centrifuged for 1 h at 100,000 \times g. The supernatant was carefully removed, 10 mM imidazole (final concentration) was added and the solution was incubated with Ni-NTA resin for 2 h. After that, the slurry was poured into a column, that was washed with Buffer B (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.1% sodium deoxycholate) supplemented with 15 mM imidazole and subsequently with Buffer C (20 mM Tris-HCl, pH 8.0, 0.5% sodium deoxycholate) supplemented with 25 mM imidazole. His_6 -GvPGHS was eluted with Buffer C supplemented with 250 mM imidazole. The fractions with the highest specific oxygenase activity (brown in color) were pooled and immediately diluted five times with Buffer D (20 mM Tris-HCl, pH 8.0, 0.3% CHAPS). The solution was incubated with DEAE-Toyopearl 650 M matrix with gentle agitation for 30 min. After sedimentation, the clear buffer solution was removed, fresh Buffer D was added and the mixture was poured into a column that was washed with 50 mM NaCl in Buffer D. His_6 -GvPGHS was eluted with 300 mM NaCl in Buffer D and stored at -70°C until used.

2.6. Gel filtration chromatography

Analytical gel filtration chromatography was conducted using a Superdex-200 10/300 GL (GE Healthcare) column mounted on an ÄKTA FPLC (GE Healthcare). The column had previously been equilibrated with Buffer E (20 mM Tris-HCl, 150 mM NaCl, 0.3% CHAPS, pH 8.0) and eluted at a flow rate of 0.5 ml/min with Buffer E. Protein elution was simultaneously monitored at 280 and 410 nm. An aliquot (20 μl) of fractions (0.5 ml) was assayed for oxygenase activity, using AA as a substrate. For the molecular mass standards, the following proteins were used: tyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa) from Pharmacia.

2.7. Electrophoretic analysis

The purity of the enzyme preparations was determined by SDS-PAGE and Coomassie Blue staining. Blue native polyacrylamide gel electrophoresis (BN-PAGE) was carried out, essentially as described previously [36]. The ionic strength of the purified protein samples was lowered using ultrafiltration through MWCO 100 kDa (Amicon, US). Protein samples were supplemented with a modified blue native sample buffer to a final concentration of 5% glycerol (w/v), 0.5% (w/v) Coomassie blue G-250 (Serva, Heidelberg, Germany), and 500 mM sodium 6-amino-*n*-caproate, incubated on ice for 60 min, and analyzed on a 5–13% polyacrylamide gradient gel.

2.8. Quantification of the purified protein

The total protein was assayed with the Lowry method using bovine serum albumin as the standard. Alternatively, proteins and albumin were separated in the SDS-PAGE and the albumin calibration curve was created according to the band intensities, using GeneTools software (Syngene). Heme content was determined spectrophotometrically (Shimadzu UV-1601) from the pyridine ferroheme assay [37], using an absorbance coefficient difference (556–538 nm) of $24.5 \text{ mM}^{-1} \text{ cm}^{-1}$. Potassium ferricyanide and sodium dithionite were used as the oxidant and the reductant, respectively.

2.9. Product analysis

Incubations of the purified GvPGHS (~1 µg) with [$1\text{-}^{14}\text{C}$]AA (a final concentration of 50 µM) were performed in 1 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM adrenaline at room temperature for 5 min. The reaction mixture was treated with 10 mM SnCl₂, acidified, and the products were extracted with ethyl acetate. The incubation products were identified by RP-HPLC and TLC, as described previously [12,38].

For the analysis of the products formed from AEA and 2-AG, 50 µg of endocannabinoid was incubated with 8 µg of protein in 1 ml of 50 mM Tris-HCl (pH 8.0), containing 1 mM adrenaline, at room temperature for 2 min. The reaction products were treated with SnCl₂ and extracted. The products were analyzed by LC-MS using a Agilent 1290 Infinity UPLC system coupled to a Agilent Technologies 6540 UHD Accurate-Mass Q-TOF LC/MS spectrometer. The MS spectra were recorded in high resolution (HR) mode.

2.10. Cyclooxygenase and peroxidase assays

Cyclooxygenase activity was determined by measuring the initial rate of oxygen uptake with a fiber optic oxygen monitor, model 110 from Instech Laboratories Inc. The kinetic experiments were conducted at 25 °C in 50 mM Tris-HCl, pH 8.0 buffer, containing 0.01% Anapoe X-100. Reactions were initiated by addition of 0.2–1.5 µg (13–100 nM) of purified enzyme to the rapidly stirring buffer containing a defined amount of substrate. The solution was pre-equilibrated against air at 1 atm. K_m and V_{max} values were determined by measuring 1–100 µM of fatty acid substrate and fitting the data to the Michaelis–Menten equation using a GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA). When the enzyme activity with 2-AG and AEA was measured 0.1% of CHAPS was used as a detergent in the reaction mixture. Peroxidase activity was evaluated spectrophotometrically (Shimadzu UV-1601) by measuring the oxidation of *N,N,N',N'*-tetramethylphenylenediamine (TMPD) at 611 nm ($\epsilon_{611} = 12,200 \text{ M}^{-1} \text{ cm}^{-1}$). Experiments were done at 25 °C in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% Anapoe X-100, 100 µM TMPD, and 0.8 µg (13 nM) of protein in a final volume of 1 ml. Reactions were initiated by adding 1–70 µM 15-HPETE or 0.05–10 mM H₂O₂ (final concentrations). 15-HPETE was prepared as described previously [39]. The initial velocity v was determined

when TMPD oxidation was linear in time. Each sample was assayed in triplicate. To determine the K_m and V_{max} values, plots of the initial velocities were used in the Michaelis–Menten equation using GraphPad Prism 5.0. k_{cat} was calculated as the maximal reaction rate (molarity of hydroperoxide reduced per second) per molar concentration of GvPGHS monomers.

2.11. Inhibition studies

The GvPGHS aliquots (equivalent to 80–90 cyclooxygenase units, where one unit is defined as 1 nmol of O₂ consumed per min at 25 °C) in 50 mM Tris-HCl (pH 8.0) containing 1 mM adrenaline were pre-incubated for 5 min at room temperature with various amounts of the inhibitor added in a few microliters of dimethyl sulfoxide. An equal amount of dimethyl sulfoxide was added to the control. Adrenaline was added to the reaction mixture to protect the enzyme from rapid loss of the activity observed at room temperature in the absence of adrenaline. When the time dependence of inhibition was determined, the pre-incubation time was extended to 20 min. The reaction was initiated with [$1\text{-}^{14}\text{C}$]AA (a final concentration of 30 µM) and carried out for 5 min. The products were extracted and analyzed using TLC, as described previously [38].

3. Results

3.1. Characterization of the algal PGHS mRNA

The PGHS related-cDNA was cloned from red algae by an RT-PCR strategy. The GvPGHS mRNA sequence was determined to consist of 2189 bp and contained a 185 bp 5'-untranslated region (UTR), a 1686 bp open reading frame and a 315 bp 3'UTR (excluding the poly(A) tail). The CtPGHS mRNA sequence was comprised of a 133 bp 5'UTR, a 1695 bp open reading frame and a 66 bp 3'UTR, with a total length of 1894 bp. The open reading frames of *G. vermiculophylla* and *C. truncatus* PGHS encode proteins of 562 and 565 amino acids with the predicted molecular masses of about 64.7 kDa and 65.5 kDa, respectively. The proteins share 57% identity in their amino acid sequences.

A comparison of deduced primary structures of *G. vermiculophylla* and *C. truncatus* PGHS with corresponding sequences of different animals, from corals to mammals, reveals 21–27% sequence identity, which is considerably lower than the average sequence identity between PGHS from different animal phyla, 60–70%. According to the primary structure, the most prominent differences between algal and animal PGHS [23,25] are the absence of an epidermal growth factor (EGF)-like domain at the N-terminal part of the algal PGHS and the lack of amino acid residues 82–95 in GvPGHS and residues 83–94 in CtPGHS, which correspond to the helix B in the membrane binding domain (Fig. 1). (The numbering of residues used here corresponds to the amino acids on ovine PGHS-1.) In contrast to deletions in the N-terminal part, the algal PGHS enzymes have several insertions larger than two amino acids in the catalytic domain after residues 321, 365, 399, 402 and 413.

Significant sequence similarities exist in the region, which corresponds to the catalytic domain of the PGHS enzyme [2,40]. In mammalian PGHS-1, Tyr-385 is noted to form a tyrosyl radical, which initiates the cyclooxygenase reaction by abstracting the 13-*pro-S* hydrogen from AA, and His-388 is noted as proximal heme ligand. These amino acid residues are essential for enzymatic activity and are thereof absolutely conserved within the PGHS family. These residues are also present in the algal sequences at the appropriate positions. In addition, Gln-203 and His-207, shown to be important for peroxidase activity, are conserved in the algal sequences. The amino acid residues essential for proper positioning of C-13 in AA for hydrogen abstraction (Tyr-348 and Gly-533), and important for PGC₂ formation through cyclization (Val-349 and Trp-387), are conserved in

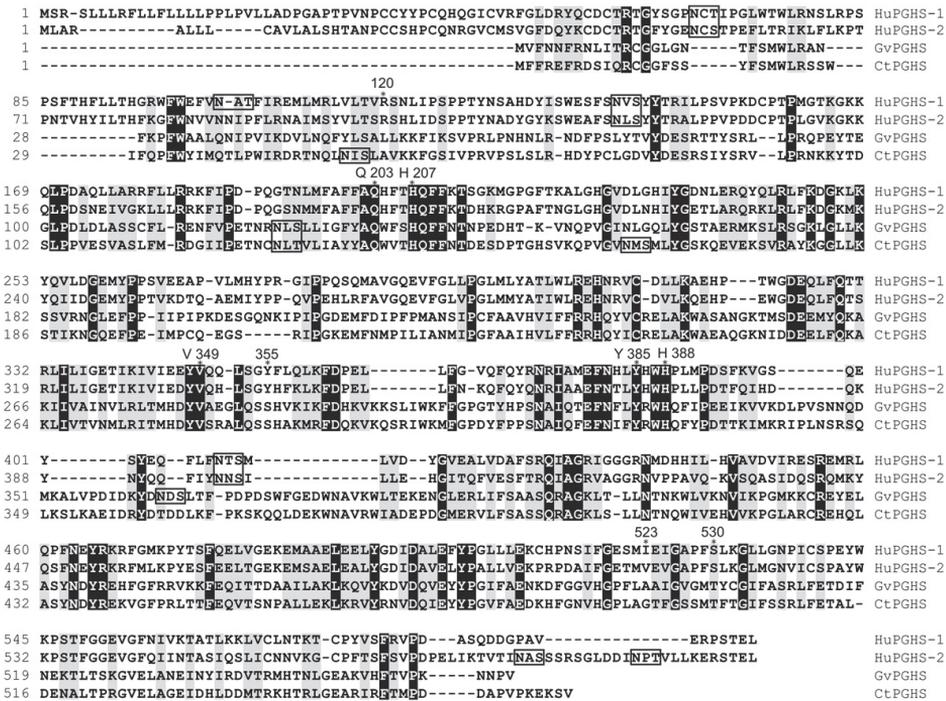


Fig. 1. Multiple sequence alignment of the deduced amino acid sequences of human and algal PGHSs. The deduced amino acid sequences of the huPGHS-1 and huPGHS-2, *Gracilaria vermiculophylla* PGHS (GvPGHS; GenBank ID: JN565603) and *Coccolytus truncatus* PGHS (CtPGHS; GenBank ID: JN565604) are compared. The sequence alignment was made using ClustalW 2.0.12. The conserved amino acid residues of PGHS proteins are highlighted with a black background, similar residues are shaded. The functionally important residues are indicated by asterisks (the ovine PGHS-1 numbering is used). The potential N-glycosylation sites are boxed.

the red algal sequences. However, the amino acid residues equivalent to Arg-120, Tyr-355 and Ser-530, shown to be important for substrate binding and coordination, and the target residues for many NSAIDs, are not found at the appropriate positions in the algal sequences.

Animal PGHS enzymes contain high mannose oligosaccharides, one of which appears to be important for proper folding [41]. GvPGHS and CtPGHS have two and three potential N-glycosylation sites, respectively, whereas their positions are not conserved between animal and algal PGHS sequences (Fig. 1). However, algal sequences do not contain signal peptides and are unlikely to be exposed to the N-glycosylation machinery.

3.2. Functional expression and purification of recombinant GvPGHS

Functional expression of algal PGHS was performed in *E. coli* BL21(DE3)RP cells. Optimal cultivation conditions were found to be 25 °C and 18 h. Expression of algal protein was verified by SDS-PAGE and activity tests. Expressions of His-tagged and untagged GvPGHS in *E. coli* cells yielded functional enzymes with comparable activities. To facilitate purification by affinity chromatography, His-tagged protein was used in further research.

Cyclooxygenase activity in the cell homogenate was found in the membrane fraction, indicating that active recombinant GvPGHS was properly integrated in membranes. A protocol that included solubilization of the membrane fraction with 1% Na-deoxycholate, and chromatography on Ni-NTA and DEAE-Toyopearl was developed for purification of recombinant GvPGHS, as described under "Materials and methods". Typical results are presented in Table 1. The Ni-NTA affinity column step yielded the most dramatic purification, increasing

the GvPGHS specific activity by ~25-fold. The DEAE-Toyopearl had little effect on the protein specific activity, but it served to concentrate the samples and remove imidazole carried over from the Ni-NTA chromatography. Overall, the purification enrichment of the recombinant GvPGHS was approximately 140-fold with a yield of 35–45% (Table 1), and typically 2–4 mg of >95% pure protein was obtained from 1 l of bacterial culture. The average specific activity of purified GvPGHS preparations was 120–130 units/mg of protein. The specific activity of the algal enzyme is higher than previously reported values for animal PGHS enzymes (e.g. 19 units/mg for huPGHS-1 [27,28], 48 units/mg for ovPGHS-1 [42] and 12 units/mg for huPGHS-2 [28]).

Table 1
Purification of recombinant His₆-GvPGHS.

Purification step	Total protein ^a mg	Specific activity ^b units/mg	Recovery %	Purification -fold
Crude extract	914	0.9	100	1
Detergent extract	143	4.7	82	5
Ni-NTA chromatography	2.9	117	41	130
Anion-exchange chromatography	2.5	124	38	138

^a Protein concentration was determined using the method of Lowry with bovine serum albumin as a standard.

^b Activity was determined using a fiber optic oxygen monitor; oxygen consumption was monitored using AA as a substrate at a concentration of 50 μM in 50 mM Tris-HCl, pH 8.0 buffer, containing 0.01% Anapoe X-100. One unit of cyclooxygenase activity is defined as 1 μmol O₂/min/mg of total protein at 25 °C in the assay mixture.

3.3. Molecular mass and oligomeric structure

Recombinant proteins obtained from the different stages of purification were subjected to SDS-PAGE (Fig. 2A). The purification yielded a nearly homogenous fraction with a single band at the molecular mass of ~63.5 kDa. The molecular mass of His₆-GvPGHS as determined by SDS-PAGE, appeared to be slightly smaller than the mass calculated from its amino acid composition (65.5 kDa).

To analyze the oligomeric state of the native His₆-GvPGHS we first used BN-PAGE with Coomassie blue G-250 as a charge shift molecule. When compared with soluble molecular mass markers (Invitrogen), the algal protein migrated at ~330 kDa (Fig. 2B). It is known that this method does not allow for an accurate estimation of the sizes of

native protein complexes, partially because the unit of mass includes the protein, variable amounts of bound Coomassie dye, and an unknown amount of detergent, and partly because in BN-PAGE the electrophoretic mobility of soluble protein markers variably deviates from that of membrane proteins of the same molecular mass [43]. However, this analysis suggested that the recombinant His₆-GvPGHS forms oligomers in CHAPS solution.

The apparent molecular mass of His₆-GvPGHS in CHAPS solution was examined by gel filtration chromatography, using a previously calibrated Superdex-200 column. The dioxygenase activity eluted from the column as a single peak with 410 nm absorption maximum, and the apparent molecular mass was determined as 268 kDa (Fig. 2C). This is approximately four-fold higher than obtained by SDS-PAGE, which led to the speculation that the native protein might consist of four subunits or two associated dimers.

When SDS-PAGE analysis was performed without treatment of a reducing agent, the band of the corresponding molecular mass did not change (data not shown), indicating that the monomeric subunits of algal PGHS are not covalently bound by disulfide bridges.

3.4. Spectral analysis and prosthetic group

The UV-visible absorbance spectrum of electrophoretically pure GvPGHS showed absorption maxima at 280 nm and at 411 nm (Soret peak). Additionally, broader but weaker maxima could be detected at 630 nm (Supplementary Fig. S1). A pyridine hemeochrome analysis of pure enzyme showed absorption maxima at 556 nm (α) and 523 nm (β), identical with a Fe(III) protoporphyrin IX standard, establishing noncovalently bound heme as the prosthetic group. The heme content of the purified recombinant GvPGHS determined by a pyridine-hemochromogen assay was 0.39 ± 0.12 mol of heme/mol of GvPGHS monomer, indicating that less than 50% of the purified protein was saturated with heme. These results are consistent with mammalian PGHSs where maximal activity occurred at a ratio of about one heme per dimer [24,26,27]. Pre-incubation of the enzyme with heme or addition of heme to the assay mixture did not affect the activity of the GvPGHS.

3.5. Identification of products

Purified GvPGHS was incubated with [^{1-¹⁴C}] arachidonic acid for 10 min. After extraction of fatty acids and their derivatives, analysis was performed by RP-HPLC and/or TLC. Recombinant GvPGHS converted AA into prostaglandin-endoperoxide, which degraded to a mixture of prostaglandins (PGF_{2 α} , PGE₂ and PGD₂). The formation of the by-products 11-HETE and 15-HETE was not observed (Fig. 3). This pattern of products is similar to that of mammalian PGHS-1 and PGHS-2 and indicates that the prostaglandins in red algae are formed via a PG-endoperoxide pathway. In animal PGHS-2, site-directed mutagenesis of Ser-530 to threonine resulted in predominant formation of prostaglandins with 15R-configuration [40,44]. The relative stereochemistry of prostaglandins formed by a GvPGHS metabolism of AA was investigated using TLC analysis with authentic standards. Prostaglandins generated by GvPGHS eluted with R_f values identical to those of 15(S)-PG standards. Under the chromatographic conditions employed, 15(R)-PG standards were readily separated from 15(S)-PG standards (data not shown). The evolutionary substitution of Ser530 to threonine in GvPGHS does not alter the configuration of the products at C-15.

3.6. Cyclooxygenase activity

Kinetic constants of the cyclooxygenase reaction were analyzed by measuring the initial rates of oxygen consumption for different substrate concentrations (five to seven concentrations in triplicates) in 50 mM Tris-HCl buffer pH 8.0 containing 0.01% detergent Anapoe

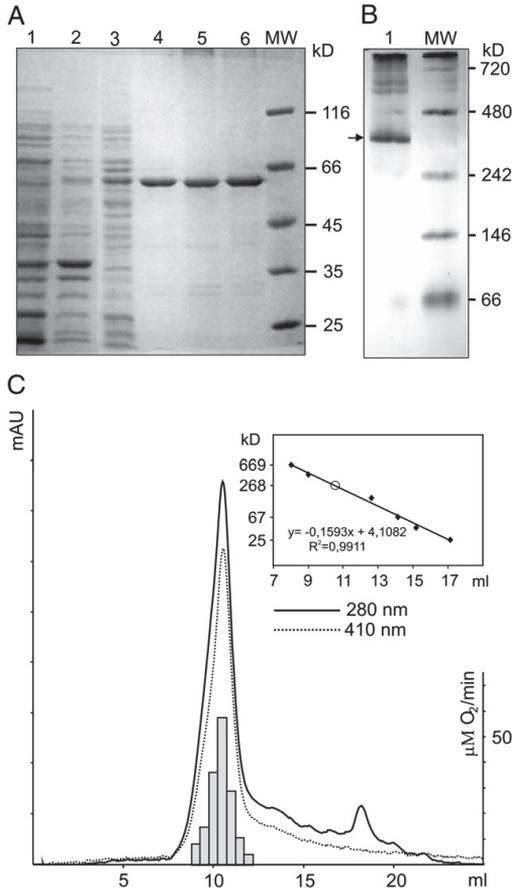


Fig. 2. Purification and analysis of His₆-GvPGHS. A. SDS-PAGE analysis of His₆-GvPGHS at different stages of purification. Aliquots after each purification step were analyzed by 10% SDS-PAGE. Lane 1, crude cell extract; lane 2, microsomes fraction; lane 3, detergent extract; lane 4, after Ni-NTA; lane 5, after DEAE; lane 6, after ultrafiltration. B. BN-PAGE analysis of His₆-GvPGHS. Purified protein in 0.4% CHAPS extract was analyzed by BN-PAGE in a 5–13% gradient gel. The apparent molecular mass of algal protein was compared with NativeMark™ molecular mass markers (Invitrogen). Oligomeric algal protein is marked by arrowhead. C. Gel filtration chromatography of His₆-GvPGHS. The analysis was conducted using a Superdex-200 (10/30GL) FPLC column. Protein elution was simultaneously monitored at 280 and 410 nm. Fractions were assayed for oxygenase activity using AA as a substrate. Activity is expressed in the bar graph as V_{opt}/20 µl of sample (µM O₂/min). Inset: Correlation between the elution volumes in gel filtration and estimated molecular masses of His₆-GvPGHS. The solid circles show size standards. The open circle shows the position of His₆-GvPGHS whose apparent molecular weight was estimated as 268 kDa.

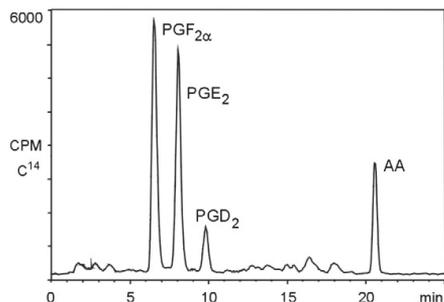


Fig. 3. RP-HPLC analysis of products formed by GvPGHS expressed in *E. coli* cells. [$1\text{-}^{14}\text{C}$]AA (50 μM) was incubated with the purified GvPGHS ($\sim 1\ \mu\text{g}$) at room temperature for 5 min, and the reaction mixture was treated with 10 mM SnCl_2 . The products were analyzed using a Zorbax Eclipse XDB-C18 5 μm column ($4.6 \times 150\ \text{mm}$) eluted at a flow rate of 1 ml/min with a mixture of acetonitrile/0.01% acetic acid at a ratio of 35/65 for 9 min, followed by linear acetonitrile increase to 100% in 9 min, and finally with 100% acetonitrile. The peaks were monitored using a Radiomatic 500TR Flow Scintillation Analyzer. The radiolabeled products were identified by co-chromatography with added unlabeled standards.

X-100. The detergent was included to minimize the side effects due to the poor solubility of the substrates and the enzyme. The reactions were started by the addition of the purified enzyme to the oxygen-saturated stirring solution. The K_m and V_{max} values were calculated by fitting the reaction rates to the Michaelis–Menten equation. The values of the kinetic parameters k_{cat} , K_m and k_{cat}/K_m are summarized in Table 2. The GvPGHS showed a preference for AA. Dihomo- γ -linolenic acid was oxygenated with lower efficiency and eicosapentaenoic acid was a poor substrate. Oxygenation of C_{18} fatty acids (linoleic acid, α -linolenic acid, and γ -linolenic acid) was not detected. The rate of the cyclooxygenase reaction (k_{cat}) of GvPGHS with AA was found to be 3–5-fold higher than values reported for mammalian PGHS enzymes [28,30,45]. On the other hand, in comparison with animal PGHSs the AA binding affinity (K_m) of algal PGHS is lower, thus yielding comparable total enzymatic efficiency (k_{cat}/K_m).

Similarly to mammalian PGHS-2, neutral arachidonate derivatives (endocannabinoids) were substrates for GvPGHS. The oxygenation rates of AEA and 2-AG by algal enzyme were 28% and 14%, respectively, compared with AA. To characterize the metabolites, the reaction products of endocannabinoids with algal enzyme were analyzed by LC-HR-MS (Supplementary Figs. S2 and S3). The product profiles formed from AEA and 2-AG by GvPGHS were similar to those formed from AA. The main products were derivatives of $\text{PGF}_{2\alpha}$ and PGE_2 .

3.7. Peroxidase activity

GvPGHS catalyzes the efficient oxidation of TMPD in the presence of hydroperoxide substrates. Similar to mammalian PGHS enzymes, the lipid hydroperoxide, 15-HPETE, is a much better substrate than

Table 2

Cyclooxygenase activity of GvPGHS. Kinetic analysis was carried out using a fiber optic oxygen monitor. Initial oxygen consumption was monitored at five to seven substrate concentrations (mean \pm S.E., $n = 3$).

Substrate ^a	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1}\ \mu\text{M}^{-1}$)
AA (20:4 ω -6)	91.6 ± 2.3	21.3 ± 1.5	4.3^b
DGLA (20:3 ω -6)	56.5 ± 1.8	22.7 ± 1.9	2.5^b
EPA (20:5 ω -3)	3.2 ± 0.1	17.3 ± 1.3	0.2
2-AG	13.0 ± 0.4	9.6 ± 1.0	1.4
AEA	25.7 ± 1.4	21.6 ± 2.9	1.2

^a Abbreviations: AA, arachidonic acid; DGLA, dihomogamma-linolenic acid; EPA, eicosapentaenoic acid; 2-AG, 2-arachidonoylglycerol; AEA, arachidonoyl ethanolamide.

^b Values were statistically different as determined by Student's *t* test ($p < 0.005$).

H_2O_2 (Table 3). The observed k_{cat}/K_m values for GvPGHS were comparable with those reported previously for the ovPGHS [42]. Surprisingly, TMPD works as a cosubstrate in a peroxidase reaction, but totally inhibits the cyclooxygenase reaction (data not shown).

3.8. Inhibition studies

Non-specific NSAIDs inhibit the cyclooxygenase activities of both PGHS-1 and PGHS-2, while PGHS-2 inhibitors called coxibs are more sensitive for PGHS-2 [28,29]. Aspirin causes covalent modification of mammalian PGHS through irreversible acetylation of a highly conserved Ser-530. The other NSAIDs inhibit enzymes by competing with AA at the cyclooxygenase active site. The effect of different non-specific NSAIDs and PGHS-2 selective nimesulide at different concentrations (0.05–1 mM) on GvPGHS activity was studied. Table 4 shows that, even at high concentrations of inhibitors, only a weak effect on algal enzyme activity was observed. This finding suggests that the conformation of the substrate binding site of the algal PGHS might be considerably different from that of the mammalian PGHS.

3.9. Characterization of genomic DNA sequences

Analysis of the red algal genomic DNA sequences revealed the lack of introns in the PGHS genes. PCR-mediated amplification of *G. vermiculophylla* and *C. truncatus* genomic DNA with primers spanning the start and the stop region of the PGHS gene (Supplementary Table S1) resulted in products with sizes identical to previously cloned cDNA sequences. A lack of introns, the unique characteristic of the genomic structure of eukaryotic red algae, has been reported in previous studies [46]. Only one PGHS gene was identified in each of the studied red algal genomes.

4. Discussion

In recent decades, genes with a certain sequence homology to animal PGHS genes have been identified in plants, fungi and bacteria. These genes encode for proteins that, in spite of some structural similarity with animal PGHSs, differ in catalytic activities: α -dioxygenases from plants convert fatty acids into 2R-hydroperoxides [47], and linoleate diol synthases from fungi oxidize linoleic acid into dihydroxyoctadecadienoic acid [48]. In this study, we characterize PGHS enzymes from red algae. The deduced amino acid sequence identity of the algal enzymes to that of PGHSs from different animals is notably low (about 20%). However, the recombinant GvPGHS is able to catalyze prostaglandin formation from polyunsaturated fatty acids.

A comparison of the predicted tertiary structure for GvPGHS with the tertiary structure observed in the crystal structure of ovine PGHS-1 suggests a conservation of overall structural architecture. As a result of the absence of an EGF-like domain, the recognizable primary structure elements appear in the algal PGHS not earlier than at position 73, which marks the beginning of the membrane binding domain helix A (Figs. 1 and 4). The latter is preceded in the algal PGHS enzymes by an 18 amino acid segment that lacks the cluster

Table 3

Peroxidase activity of GvPGHS. Formation of oxidized TMPD was monitored spectrophotometrically at 611 nm. The initial rates of reduction of hydroperoxide substrate were calculated from the rates of TMPD oxidation (mean \pm S.E., $n = 3$).

Peroxide	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1}\ \mu\text{M}^{-1}$)
15-HPETE ^a	31 ± 0.8	6.7 ± 0.6	4.7
H_2O_2	59 ± 1.6	3309 ± 216	1.8×10^{-2}

^a Abbreviations: 15-HPETE, 15-hydroperoxyicosatetraenoic acid.

Table 4

Inhibition of GvPGHS activity. The GvPGHS aliquots in 50 mM Tris–HCl (pH 8.0) were pre-incubated for 5 min at room temperature with various amounts of the inhibitor. The remaining activity was then determined using [3 H]AA (30 μ M) as a substrate. The products were extracted and analyzed by TLC. Means \pm S.D. (n = 3) are shown.

Inhibitor	Concentration	Remaining activity
	mM	%
Indomethacin	0.2	87 \pm 12
	1	71 \pm 18
Flurbiprofen	0.2	81 \pm 23
	1	57 \pm 11
Nimesulide	0.2	90 \pm 7
	1	83 \pm 7
Aspirin	5	97 \pm 27
	10	61 \pm 13

of hydrophobic amino acid residues characteristic of eukaryotic signal peptides. According to the predicted model, the overall topology of secondary structure elements in the catalytic domain of algal PGHS closely resembles that of animal PGHS enzymes (Fig. 4) and, as long as both major deletions in the algal PGHS (EGF-like domain and helix B in the membrane binding domain) have occurred well outside the catalytic domain, a similar catalytic mechanism with animal PGHS can be expected. Moreover, all amino acid insertions in GvPGHS are invariably located in the peripheral regions of the catalytic domain, forming mostly unstructured loops and, consequently, the impact of these insertions on the catalytic mechanism should be minimal (Fig. 4).

Differences in amino acid residues forming the first and second shell of the cyclooxygenase active site channel [40,49] are more noticeable: on average ~46% of residues are conserved between algal and animal PGHS, while the respective number between different animal phyla is ~89%. The overall geometry of the cyclooxygenase active site of GvPGHS resembles more that of PGHS-2. A highly conserved difference between the PGHS isoforms is the presence of Val-523,

Arg-513, and Val-434 in PGHS-2, as opposed to Ile-523, His-513, and Ile-434 in PGHS-1. The smaller side chains in PGHS-2 form a cavity, called the “side pocket”, which increases the volume of the active site [31]. GvPGHS has the amino acid residues Leu-434, Asp-513 and Ala-523 at appropriate positions and the formed “side-pocket” is even more spacious than that of animal PGHS-2.

Perhaps the most interesting substitutions in GvPGHS compared to animal PGHS are at Arg120Leu, Tyr355Ser and Ser530Thr (Fig. 1). Arg-120 has been shown to be critical in binding the substrate fatty acid carboxylic group via an ionic bond in PGHS-1 [50], but is dispensable in the case of PGHS-2 [45,51], which could explain the ability of algal PGHS to oxidize substrate fatty acids in spite of having Leu-120 at the appropriate position. Arg120Leu substitution is likely responsible for the insensitivity of GvPGHS toward non-steroidal inhibitors containing a carboxylic acid moiety such as flurbiprofen and indomethacin, which did not inhibit algal PGHS activity up to concentrations of 200 μ M (Table 4), although it has been shown that Arg120Leu mutation is not equally effective at eliminating inhibition by indomethacin in the case of PGHS-2 [52,53].

Tyr-355 is located in an unstructured loop after helix H6 and its hydroxyl group interacts via a hydrogen bond with the carboxylic group of substrate fatty acids and NSAIDs [40,51]. Tyr355Ser substitution in GvPGHS most likely abolishes the aforementioned interactions. According to the model, the smaller sterical hindrance of the side chain of serine residue compared to that of tyrosine allows for the displacement of the peptide chain, resulting in the facing of the hydroxyl group of Ser-355 away from the cyclooxygenase active center. Interestingly, a compensatory Leu531Tyr substitution at the lower part of the cyclooxygenase active center, accompanied by Ala527Val substitution, may have resulted in a substrate conformation, where Tyr-531 provides a hydrogen bonding capability, while Val-527 interacts with carbons C6 and C7. These substitutions plausibly force the carbon C2 of the substrate fatty acid towards the larger “side-pocket” resulting from Ala-523 at the appropriate position, and shift the carbons C6 and C7 towards the helix H6. Differences in the geometry of the cyclooxygenase active center of GvPGHS may

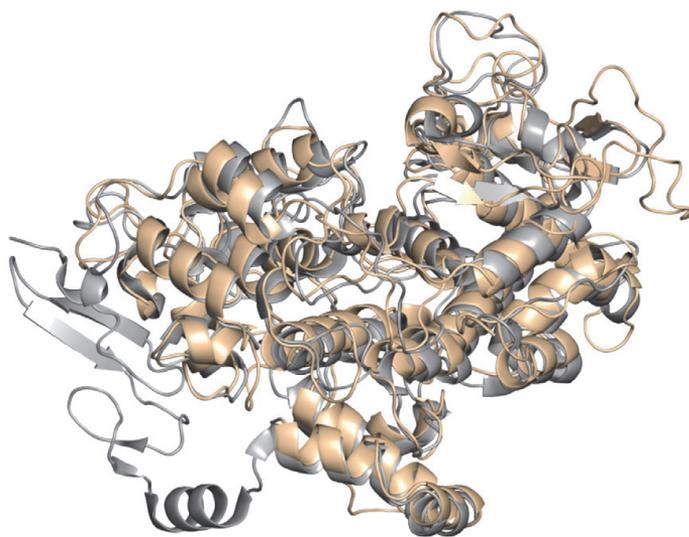


Fig. 4. Superposition of ovine PGHS-1 X-ray structure (1DIY) and modeled GvPGHS structure. The superposition of animal (gray) and algal (beige) PGHS structures shows a generally similar arrangement of secondary structure elements and indicates insertions and deletions in comparison of two enzymes. Modeling was conducted using a CPHModels 3.0 algorithm. The model was built using 1DIY from <http://www.rcsb.org> as a template.

also account for the insensitivity towards several NSAIDs tested, as well as for the ability to oxidize several fatty acid derivatives, such as 2-AG and AEA.

Ser-530 is located just below of the tyrosyl radical forming Tyr-385 at the opposite side of the cyclooxygenase active center, and is the site of acetylation by aspirin [23]. Ser530Thr substitution in GvPGHS has effectively rendered the enzyme insensitive to aspirin up to concentrations of 5 mM, while the product profile of the algal PGHS enzyme has not changed, unlike the respective mutants of animal PGHS [40,44]. These unique properties of GvPGHS likely arise from the aforementioned compensatory mutations, especially Leu531Tyr substitution adjacent to Ser-530. Because of the greater steric volume of the tyrosine side chain compared to that of leucine, a substrate fatty acid plausibly achieves a catalytically productive, yet alternative conformation, thus enabling the formation of 15S-PGG₂ in spite of having Thr-530 at the corresponding position.

Curiously, with the loss of the EGF domain, the C-terminal disulphide bond between residues 569 and 575 also disappeared from algal PGHS. Moreover, GvPGHS seems to have no signal peptide present at the N-terminus, and it has been shown that, though there are two N-glycosylation consensus sequences at positions 193 and 406, the enzyme does not need N-glycosylation for catalytic activity. GvPGHS was functionally expressed in the prokaryotic system and not directed to the N-glycosylation machinery when heterologously expressed in eukaryotic COS-7 cells (data not shown).

The absence of a signal peptide and the EGF domain, together with the loss of the need for N-glycosylation, raise questions about the sub-cellular localization of algal PGHS enzymes as long as both of them have Cys569Arg and Cys575Glu substitutions replacing the disulphide bond with an ionic one to preserve a critical function that is absolutely conserved in all animal PGHS known so far. One can speculate that the algal PGHS shares a common ancestry with animal PGHS enzymes and evolved differently after the divergence of algal and animal lineages, preserving more primordial features characteristic of ancestral enzyme, while animal PGHS enzymes have acquired novel structural determinants in the course of evolution. Thus, the algal PGHS enzymes offer new insights into eicosanoid biochemistry and perhaps will shed some light on the evolutionary origins of prostaglandin synthesis.

In conclusion, the first non-animal PGHS from marine red algae displays atypical structural and catalytic features. Differently from animal PGHSs the GvPGHS easily expresses in *E. coli* as a catalytically highly active enzyme. Algal PGHS metabolizes not only polyunsaturated free fatty acids but also ester and amide derivatives of AA (AEA and 2-AG) to corresponding prostaglandin products. Algal PGHS is not inhibited by non-steroidal anti-inflammatory drugs. The algal enzyme lacks some structural elements identified in all known animal PGHSs, e.g. EGF-like domain and helix B of the membrane binding domain, and certain residues shown to be important for substrate binding and coordination. These differences in the primary structure are significant and therefore may cause bigger conformational changes in the membrane binding domain and in the entry to the substrate binding channel than speculated on the bases of modeling data. Crystallographic studies of algal PGHS, preferentially complexed with substrates, may provide further information about conformations and interactions in this part of the protein.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbali.2012.11.010>.

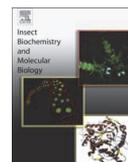
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Direct evidence of the cyclooxygenase pathway of prostaglandin synthesis in arthropods: Genetic and biochemical characterization of two crustacean cyclooxygenases[☆]

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ABSTRACT

Prostaglandins, well-known lipid mediators in vertebrate animals, have also shown to play certain regulatory roles in insects and other arthropods acting on reproduction, immune system and ion transport. However, knowledge of their biosynthetic pathways in arthropods is lacking. In the present study, we report the cloning and expression of cyclooxygenase (COX) from amphipod crustaceans *Gammarus* spp and *Caprella* spp. The amphipod COX proteins contain key residues shown to be important for cyclooxygenase and peroxidase activities. Differently from all other known cyclooxygenases the N-terminal signal sequence of amphipod enzymes is not cleaved during protein expression in mammalian cells. The C-terminus of amphipod COX is shorter than that of mammalian isoforms and lacks the KDEL(STEL)-type endoplasmic reticulum retention/retrieval signal. Despite that, amphipod COX proteins are N-glycosylated and locate similarly to the vertebrate COX on the endoplasmic reticulum and nuclear envelope. Both amphipod COX mRNAs encode functional cyclooxygenases that catalyze the transformation of arachidonic acid into prostaglandins. Using bioinformatic analysis we identified a COX-like gene from the human body louse *Pediculus humanus corporis* genome that encodes a protein with about 30% sequence identity with human COX-1 and COX-2. Although the COX gene is known to be absent from genomes of *Drosophila* sp., *Aedes aegypti*, *Bombyx mori*, and other insects, our studies establish the existence of the COX gene in certain lineages within the insect world.

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1. Introduction

Prostaglandins are formed by the action of cyclooxygenase (COX; EC 1.14.99.1) that catalyzes the committed step in the conversion of arachidonic acid to the prostaglandin-endoperoxides PGG₂ and PGH₂ (Rouzer and Marnett, 2003; van der Donk et al., 2002). The COX genes have been identified in all the vertebrates investigated, including

mammals, birds, teleosts, and cartilaginous fishes. In vertebrates there are at least two COX isozymes that are encoded by distinct genes: the constitutively expressed COX-1 and inducible COX-2. These proteins share about 60–65% amino acid sequence identity within species, the sequence identity among orthologs from different species is even higher, being 85–90% (Smith et al., 2000). The occurrence of prostaglandins and other eicosanoids has also been reported in several marine invertebrate species, like corals, crustaceans, mollusks and chordates (reviewed by Gerwick et al., 1993; Gerwick, 1999; Rowley et al., 2005). The prostaglandin-endoperoxide pathway of prostaglandin biosynthesis in non-vertebrate animals was first established in the arctic soft coral *Gersemia fruticosa* (Varvas et al., 1994). Later, the COX enzymes sharing about 50% amino acid identity with both vertebrate COX-1 and COX-2, were cloned and characterized in the corals *G. fruticosa* and *Plexaura homomalla* (Koljak et al., 2001; Valmsen et al., 2001, 2007; Järving et al., 2004).

The biological role of prostaglandins and other eicosanoids has also been reported in terrestrial arthropods, particularly in insects

Abbreviations: COX, cyclooxygenase; PG, prostaglandin; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulphate; UTR, untranslated region; ER, endoplasmic reticulum; HETE, hydroxyeicosatetraenoic acid; HHT, 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid; RP-HPLC, reversed-phase high pressure liquid chromatography; EST, expressed sequence tags; Pxt, peroxinectin; MPO, myeloperoxidase; Pxd, peroxidase.

[☆]Data deposition: The sequences reported in this paper have been deposited in the GenBank database under accession nos. GQ180795 (caprellid COX) and GQ180796 (gammarid COX).

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(reviewed by Stanley, 2000, 2006; Stanley and Miller, 2006) and in aquatic arthropods (reviewed by Rowley et al., 2005). Classical prostaglandins, particularly PGE₂, PGD₂, and PGF₂α, have been detected in prawns *Macrobrachium rosenbergii* (Sagi et al., 1995) and *Marsupenaeus japonicus* (Tahara and Yano, 2004), as well as in crabs *Carcinus maenas* (Hampson et al., 1992) and *Oziotelphusa senex senex* (Reddy et al., 2004). These studies provide evidence that prostaglandins are involved in the regulation of reproduction in crustaceans. However, the mode of prostaglandin biosynthesis in arthropods and enzymes involved in these pathways has remained unresolved. Even more, the homologues of mammalian COX genes have not been identified in completely sequenced insect genomes of *Drosophila* sp., *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, *Tribolium castaneum* and others. The only bioinformatic evidence of the possible COX pathway in arthropods is derived from crustaceans (Heckmann et al., 2008). Recently it was shown that exogenous prostaglandins mediate the oogenesis of *Drosophila* and that a peroxidase Pxt may function as a *Drosophila* COX during follicle maturation (Tootle and Spradling, 2008). Pxt is a heme peroxidase with residues, which are shown to be crucial for heme coordination and peroxidase activity. However, critical residues for cyclooxygenase activity and substrate binding like Arg120, Tyr355 and Tyr385 are not clearly conserved in Pxt and there is no evidence of the occurrence of C20 polyunsaturated fatty acids in *Drosophila melanogaster* lipids either (Pages et al., 1986; Overgaard et al., 2005).

As crustaceans living in an aquatic environment have higher proportions of prostaglandin precursor fatty acids in their phospholipids than most terrestrial insects (Stanley, 2000; Sushchik et al., 2003), they are a good material for studies of prostaglandin biosynthesis in arthropods. In the present study, we describe molecular cloning and characterization of cyclooxygenase-encoding genes in amphipod crustaceans. The cyclooxygenases identified have a high homology to mammalian COX-1 and COX-2 in the active site, but significant differences in the N- and C-termini of the protein. In spite of these differences, the amphipod COX mRNAs encode functional cyclooxygenases that catalyze the transformation of arachidonic acid into prostaglandins. In addition, by using database searches, we constructed and analyzed the sequences and exon/intron structures of COX genes from human body louse *Pediculus humanus corporis* and cladoceran crustacean *Daphnia pulex*.

2. Experimental procedures

2.1. Materials

The crude samples were collected from the coast of Kanagawa prefecture in Tokyo Bay in March 2003. The samples contained red algae *Gracilaria asiatica* (Rhodophyta) and small amphipod crustaceans *Gammarus* spp and *Caprella* spp (Crustacea: Amphipoda) inhabiting the macroalgae community. The samples were transported and stored at –80 °C.

2.2. Preparation of RNA and cDNA synthesis

Total RNA from crude samples was prepared as described previously (Koljak et al., 2001). mRNA was prepared from the total RNA by using an oligo(dT)-cellulose column and mRNA purification kit (QIAGEN). The first strand cDNA was prepared using an oligo(dT)-adaptor primer (Song et al., 1993).

2.3. PCR-cloning

Initial PCR fragments were obtained using the degenerate primers based on the conserved regions of known COX sequences as described previously (Koljak et al., 2001) with light

modifications. An additional upstream degenerate primer based on the conserved CDCTRTG sequence was used in PCR experiments. 5'-RACE was accomplished using a 5'-RACE kit (Roche Diagnostics) according to the manufacturer's instructions. The oligonucleotide sequences used in this study are given in Table 1 (Supplementary material). 3'-RACE was accomplished using the first strand cDNA prepared with an adaptor linked oligo(dT) primer (Koljak et al., 2001). All the PCR products were cloned into the pGEM-T Easy vector (Promega), amplified in *E. coli*, and sequenced.

Simultaneously, the crude sample that contained red algae and amphipods was thawed, individual animals were separated, washed with sterile water, and genomic DNA was extracted from algal and amphipod species, using a simple SDS method. Briefly, the washed individual animals and small algal pieces were transferred into separate tubes and 50–100 µl of 0.25% SDS solution was added. The probes were incubated at 90 °C for 15 min with a gentle mixing. The mixture was cooled to room temperature and centrifuged at 13 000 × g for 2 min. The supernatant containing the genomic DNA was transferred to a fresh tube and used as a template in PCR reactions with gene-specific primers. It was verified that the COX sequences identified were derived from amphipod crustaceans *Gammarus* spp and *Caprella* spp and not from any other material.

2.4. Full-length cDNA clones

cDNA for the full-length amphipod COX proteins were obtained by PCR using the proofreading *Pfu* polymerase (Promega) and 1 µl of the first strand cDNA as a template. For *gammarid* COX primers DTMN-up and GTL-down (Supplementary: Table 1) with the *HindIII* site added at the 5' end of both primers to facilitate subcloning were used. For *caprellid* COX primers AMSD-up and GTP-down with the *BamHI* site added at the 5' end of both primers were used. The reaction conditions were 1 cycle at 95 °C for 2 min; 10 cycles at 95 °C for 30 s, 50 °C for 45 s (for *gammarid* COX) or 55 °C for 45 s (for *caprellid* COX), 72 °C for 4 min; 20 cycles at 95 °C for 30 s, 50 °C (for *gammarid* COX) or 55 °C (for *caprellid* COX) for 45 s, 72 °C for 4 min + 20 s for each cycle; and 1 cycle 72 °C for 10 min. The ends of the PCR products were digested with *HindIII* or *BamHI*, respectively. The digested products were purified on agarose gel and cloned into the pCG-E2Tag vector (Kaldalu et al., 2000) for a transient expression in COS-7 cells. The plasmid DNA was isolated and purified using the Qiagen plasmid purification system. Two clones of the COX constructs of both amphipods were full-length sequenced and expressed. The coral *G. fruticosa* COX-A and rabbit COX-2 constructs in pCG-E2Tag vectors were used as positive controls in transfections for expression in COS-7 cells. The rabbit COX-2 cDNA was isolated from the RabCOX-2-pcDNA3.1 construct (a kind gift from Dr. Matthew Breyer, Vanderbilt University).

2.5. Isolation of genomic DNA

For gene structure studies the genomic DNA was isolated using a modification of the CTAB procedure as described previously (Järving et al., 2004). The crude sample was thawed and individual animals were separated, washed with sterile water and transferred into 0.5 ml or 1 ml of the prewarmed (65 °C) isolation buffer (2% w/v CTAB, 1.4 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH8.0), 0.2% β-mercaptoethanol). Proteinase K (100 µg/ml final concentration) was added and the tube was incubated at 65 °C for 2 h. Almost all the material was dissolved. The solution was then twice extracted with equal volumes of chloroform/isoamyl alcohol (24:1, v/v) and centrifuged at 2000 × g for 5 min. The final aqueous phase was transferred to a new tube and 0.6 vol of cold (–20 °C) isopropanol was added. The mixture was kept at –20 °C overnight, subsequently the genomic DNA was precipitated by centrifugation at 2000 × g for 5 min at 4 °C. The precipitate was twice washed with

a cold wash solution containing 10 mM ammonium acetate and 70% ethanol. The genomic DNA pellet was air dried and dissolved in 50 μ l of TE buffer and stored at +4 °C.

2.6. Amplification and cloning of genomic DNA

Genomic DNA sequences were amplified by PCR using the Expand Long Template PCR System with buffer 3 (Roche Diagnostics), 0.5 mM each dNTP, 0.3 μ M gene-specific primers, and 1 μ l template DNA solution in reactions with a final volume of 50 μ l. The gene-specific primers were constructed using mRNA data. To distinguish the mRNA products from genomic products the forward and reverse primers were selected from distinct putative exons that bridge intervening introns (Supplementary: Table 1). The PCR program was 1 cycle at 94 °C for 2 min; 10 cycles at 93 °C for 30 s, 53 °C for 45 s, 68 °C for 10 min; 25 cycles at 93 °C for 30 s, 53 °C for 45 s, 68 °C for 10 min + 20 s for each cycle, and 68 °C for 15 min. The PCR products were cloned into the pGEM-T Easy vector, amplified in *E. coli*, and the genomic DNA sequences were compared with the cDNA sequences.

2.7. DNA sequencing and bioinformatics

The clones were sequenced using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) and an ABI 3130 Genetic Analyzer, or the sequencing was carried out by AGOWA (Berlin, Germany). Sequence alignments were obtained employing the Clustal method using the Lasergene program (DNASTar, Inc.). The signal peptide cleavage site was predicted using SignalP version 3.0 (Bendtsen et al., 2004). The primary amino acid sequences from various organisms were obtained from the GenBank and Joint Genome Institute databases (<http://www.jgi.doe.gov>). *D. pulex* sequence data were obtained from <http://wflabase.org>. The phylogenetic tree and graphic presentation were generated online (<http://align.genome.jp>) using multiple sequence alignment ClustalW program with default parameters (Thompson et al., 1994).

2.8. Cells and transfections

The COS-7 cells were maintained in Iscove's modified Dulbecco's medium with 10% fetal bovine serum. The transfections were performed as described previously (Koljak et al., 2001). The tunicamycin treatments of COS-7 cells transfected with gammarid pCG-E2Tag-COX, caprellid pCG-E2Tag-COX, and *G. fruticosa* pCG-COX-A(E2Tag) constructs were performed by adding tunicamycin (Sigma) to the media at final concentration of 1 μ g/ml 4 h after transfection. The cells were grown at 37 °C and harvested at 28 h post-transfection.

2.9. Immunoblot analysis

The COS-7 cells transfected with expression plasmids were lysed 48 h after electroporation in 100 μ l of the Laemmli sample buffer. The proteins were separated by SDS-9% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were incubated with anti-E2Tag mouse monoclonal antibody (Quattromed, Estonia) or rat COX-2 specific mouse monoclonal antibody (PharMingen) and with secondary horseradish peroxidase-conjugated antibody (LabAs, Estonia) according to the manufacturer's recommendations. Detection was performed using an ECL detection kit (Amersham Biosciences).

2.10. Immunofluorescence analysis

For immunofluorescence analysis, the COS-7 cells transfected with an appropriate expression vector were grown on coverslips at

37 °C for 24 h and fixed in cold (–20 °C) acetone/methanol (1:1) for 15 min. The coverslips were washed with PBS and blocked with bovine serum albumin (1 mg/ml in PBS) for 30 min. The anti-E2Tag monoclonal antibody was added at a concentration of 5 ng/ μ l (in bovine serum albumin/PBS) or anti-COX-2 monoclonal antibody at a dilution of 1:100, and both were incubated at room temperature for 1 h and washed with PBS. As a secondary antibody, the Alexa Fluor 488 conjugated goat anti-mouse IgG antibody (1:1000; Invitrogen) was used. The results were analyzed using a confocal Nikon Eclipse TE2000-U microscope and the cells with similar signal intensities were captured.

2.11. Microsome preparation

The transfected cells were grown at 37 °C for 40 h, harvested with PBS/3 mM EDTA, and collected by centrifugation at 1000 \times g for 5 min. The cells (2×10^6) were then resuspended in 1 ml of buffer M (50 mM Tris–HCl (pH 8.0), containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT) and disrupted by sonication 3×5 s each time, 50% time interval, 60% capacity (Sonopuls, Bandelin) on ice. Then 2 ml of buffer M was added and the nuclei and other particulate cellular components were separated by centrifugation at 3000 \times g for 10 min. The supernatant was transferred to a new tube and centrifuged at 200,000 \times g for 1 h. The soluble cytoplasmic proteins of the supernatant (fraction S) were precipitated with trichloroacetic acid (final concentration 10% and 0.01% NaDoc) and suspended in 40 μ l of the Laemmli sample buffer. The pellets from the first (fraction N, nuclei) and second (fraction M, microsomal membranes) centrifugations were resuspended in 40 μ l of the Laemmli sample buffer. Each fraction was subjected to immunoblot analysis.

2.12. Enzyme assay and product analysis

For activity assay, the transfected COS-7 cells were grown at 28 °C for 72 h (Koljak et al., 2001). The cells were harvested and the microsomal fraction was prepared as described before (Järving et al., 2004). Incubations of the microsomal fraction of transfected cells ($4\text{--}5 \times 10^6$ cells/incubation) with [$1\text{-}^{14}\text{C}$]arachidonic acid (final concentration of 50 μ M) were performed in 1 ml of 50 mM Tris–HCl (pH 8.0) containing 1 mM adrenaline and 1 μ M hemin at room temperature for 10 min. The reaction mixture was treated with 10 mM SnCl₂, acidified to pH 3, and the products were extracted with ethyl acetate. For product identification the RP-HPLC analysis was performed using a Rigel ODS 5- μ m column (4.6 \times 250 mm) eluted at a flow rate of 1 ml/min with a stepwise gradient of acetonitrile/0.017 M H₃PO₄ mixture at a ratio of 35/65, v/v for 9 min and 70/30, v/v for 9 min, and finally with 100% acetonitrile. The peaks were monitored using a Radiomatic 500TR Flow Scintillation Analyzer (Packard Bioscience). Prior to HPLC analysis the unlabeled standards of prostaglandins (a generous gift from Kevelt, Estonia), HETE and HHT (Cayman Chemical) were added to facilitate the UV detection of peaks.

2.13. Inhibition studies

The microsomal fraction of transfected cells ($2\text{--}4 \times 10^6$ cells/incubation) in 50 mM Tris–HCl (pH 8.0) containing 1 μ M hemin and 1 mM adrenaline was preincubated at room temperature for 5 min with various amounts of the inhibitor added in a few microliters of ethanol. An equal amount of ethanol was added to the control. The reaction was initiated with [$1\text{-}^{14}\text{C}$]arachidonic acid (final concentration of 30 μ M). The incubation was carried out for 10 min then terminated. The products were extracted and analyzed using TLC as described previously (Varvas et al., 1999).

The 3'UTR sequence of gammarid COX is almost twice longer than that of caprellid COX and contains four polyadenylation signals AAUAAA whereas the gene of caprellid COX contains one polyadenylation signal (boxed in Fig. 1B). The 3'UTR sequences of both genes are AU-rich (70% of A and U residues). Vertebrate COX-2 mRNAs contain AU-rich elements (AREs) and multiple copies of the mRNA instability AUUUA motif in their 3'UTR sequences (Dixon et al., 2000; Cok and Morrison, 2001). The presence of AREs is common characteristic of immediate-early genes and is implicated in the post-transcriptional regulation of mRNA stability (Shaw and Kamen, 1986; Espel, 2005). Gammarid and caprellid COX mRNA sequences possess six and five AUUUA sequences, respectively (Fig. 1B). The presence of AREs in their 3'UTR sequences suggests that both amphipod COX genes might be inducible genes.

3.2. Expression of amphipod COX proteins in COS-7 cells

The amphipod COX proteins differ significantly in structure from other known COX enzymes. The differences relate to the N- and C-terminal ends of proteins including signaling sequences and to the pattern of potential N-glycosylation sites. To investigate how these differences could alter the properties of the protein both amphipod cyclooxygenases were heterologously expressed in COS-7 cells.

3.2.1. Signal sequences

COX-1 and COX-2 have an N-terminal sequence characteristic of a cleavable signal peptide for translocation into an endoplasmic reticulum (ER) lumen and both COX isoforms are processed into mature forms by removal of signal peptides (Kulmacz et al., 2003). The *in silico* analysis (SignalP 3.0) of amphipod cyclooxygenases predicted signal sequences with low cleavage probability. To verify the retention of the signal sequence during maturation of proteins the open reading frames of gammarid and caprellid COX were cloned into the eukaryotic expression vector pCG in the frame with the N-terminal epitope tag – E2Tag (Fig. 2A), transfected into COS-7 cells and analyzed 48 h after transfection. The coral *G. fruticosa* COX-A and rabbit COX-2 were used as positive controls. To follow the cleavage of the predicted N-terminal signal peptide we analyzed the transfected cells by immunoblot analysis using two different antibodies – the anti-E2Tag antibody recognizing epitope tag in front of the signal peptide, and the COX-specific antibody. As shown in Fig. 2B, the anti-E2Tag monoclonal antibody recognized the gammarid and caprellid E2Tag-COX proteins (lanes 1 and 2) and the coral COX-A(E2Tag) protein (lane 4), in which the tag was fused in the middle of the protein, but was not able to recognize the coral and rabbit COX proteins in which E2Tag was N-terminally fused (lanes 3 and 5). At the same time, the coral and rabbit COX proteins were readily detectable by a COX-2-specific antibody (lanes 3–5). The rat COX-2 specific mouse monoclonal antibody reacted specifically with the caprellid COX, but not with the gammarid COX. From these experiments we can conclude, that unlike all other known COX proteins, the signal sequence of amphipod COX is not cleaved during processing to the mature protein in mammalian cells.

3.2.2. Subcellular targeting

The C-termini of both amphipod COX are shorter by 13 residues than those of mammalian COX-1 and shorter by 31 residues than those of COX-2 and lack a KDEL(STEL)-type ER retention/retrieval signal as well (Fig. 1A). The C-terminus of the catalytic domain of mammalian COX-1 and COX-2 contains modified versions of the ER targeting KDEL sequence, and COX-2 has an additional 18-amino acid sequence located next to this retention signal (Kulmacz et al., 2003). To determine the localization of amphipod COX proteins within the cells an indirect immunofluorescence microscopy

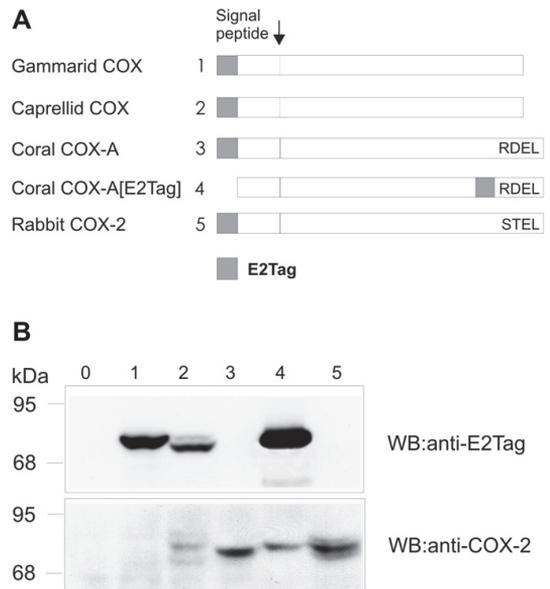


Fig. 2. Expression of gammarid and caprellid COX in COS-7 cells. (A) Schematic presentation of the epitope-tagged COX proteins used in this study. (B) Immunoblot analysis of COS-7 cells transfected with the gammarid pCG-E2Tag-COX (lane 1), the caprellid pCG-E2Tag-COX (lane 2), the coral *G. fruticosa* pCG-E2Tag-COX-A (lane 3), the *G. fruticosa* pCG-COX-A[E2Tag] (lane 4), and the rabbit pCG-E2Tag-COX-2 (lane 5) expression vectors or carrier DNA (negative control, lane 0). 48 h after transfection, cell extracts were prepared and analyzed by immunoblotting with anti-E2Tag antibody or anti-COX-2 antibody as described under "Materials and Methods".

analysis was used. The COS-7 cells, transfected with expression plasmids encoding the gammarid E2Tag-COX, caprellid E2Tag-COX, coral COX-A(E2Tag), and rabbit COX-2, showed in all cases an intense peri-nuclear ring and a strong signal associated with subcellular structures, probably the ER of the cell (Fig. 3A). The fluorescence signal was excluded from the nuclear space.

To verify whether the amphipod COX proteins were membrane-associated, we examined the distribution of recombinant proteins in the particulate and soluble fractions of COS-7 cells expressing gammarid COX and caprellid COX proteins. The transfected cells were disrupted 40 h after transfection by sonication and separated into three subcellular fractions that corresponded roughly to nuclei, microsomal membranes, and soluble cytoplasmic proteins. As shown in Fig. 3B, the amphipod COX proteins fractionated with the microsomal and nuclear membrane fractions, similarly to the rabbit and coral COX proteins. It has been demonstrated that mammalian COX-1 and COX-2 are integral membrane proteins that are associated exclusively with the nuclear pellet and microsomal fractions (Spencer et al., 1998). Thus, the cellular localization of amphipod COX proteins is similar to that of the mammalian counterparts in spite of differences in N- and C-termini of these proteins.

3.2.3. N-linked glycosylation

The proper folding and activity of COX enzymes depend on the N-linked glycosylation (Otto et al., 1993; Kulmacz et al., 2003). Gammarid and caprellid COX have four and three potential N-glycosylation sites, respectively (Fig. 1A). The positions at Asn68 and Asn144 are conserved between amphipod and mammalian COX, but the third potential N-glycosylation site is at Asn213 and the gammarid COX has an additional consensus site at Asn439. To determine

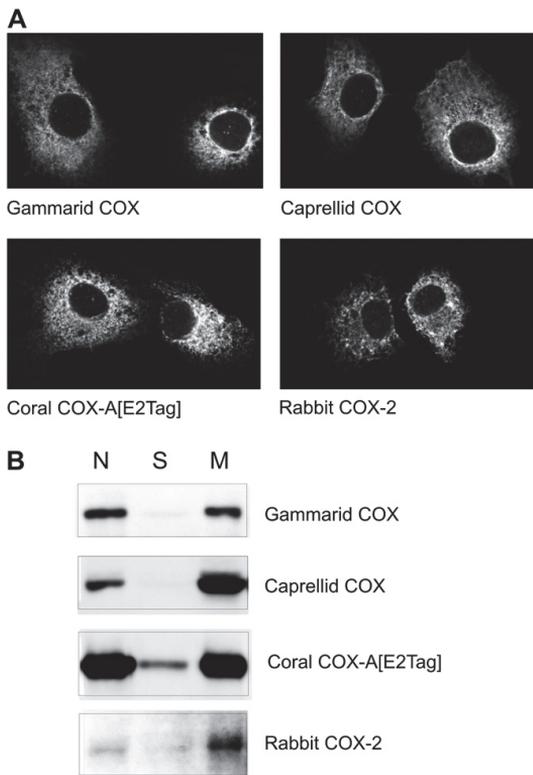


Fig. 3. Subcellular localization of gammarid COX, caprellid COX, coral COX-A[E2Tag], and rabbit COX-2 in COS-7 cells. (A) Immuno-fluorescence staining of COS-7 cells expressing various COX variants. COS-7 cells were grown on coverslips for 24 h, then fixed and incubated with anti-E2Tag antibody or anti-COX-2 antibody, followed by incubation with Alexa Fluor 488 conjugated goat anti-mouse IgG antibody. (B) Immunoblot analysis of nuclear, microsomal, and soluble protein fractions from transfected COS-7 cells. 40 h after transfection, the cells were collected and the fractions containing nuclear and other particulate organelles (N), soluble proteins (S), and microsomal membranes (M) were prepared and analyzed by immunoblotting with anti-E2Tag antibody or anti-COX-2 antibody.

whether amphipod COX proteins are glycosylated, the expression of the amphipod COX in COS-7 cells was performed in the presence or absence of the N-glycosylation inhibitor, tunicamycin. The treatment with tunicamycin reduced the apparent molecular weight of gammarid and caprellid COX as was the case with the coral *G. fruticosa* COX-A that has been shown to be glycosylated (Järving et al., 2004) (Fig. 4). These results demonstrate that the amphipod proteins are glycosylated.

3.3. Cyclooxygenase activity of amphipod COX proteins

In order to test the enzymatic activity of amphipod COX proteins the transfected COS-7 cells were grown at 28 °C for 72 h. The microsomal fractions were prepared and incubated with [14 C]arachidonic acid. The incubation products were analyzed using RP-HPLC. Both amphipod recombinant cyclooxygenases converted arachidonic acid into the prostaglandin-endoperoxide, which degraded to a mixture of prostaglandins (PGF $_{2\alpha}$, PGE $_2$ and PGD $_2$) together with the by-products HHT and 11-HETE. The formation of 15-HETE was not observed (Fig. 5).

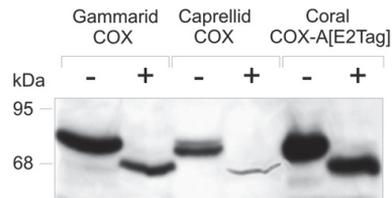


Fig. 4. Immunoblot analysis of gammarid COX, caprellid COX, and coral COX-A[E2Tag] expressed in COS-7 cells with and without the presence of tunicamycin. COS-7 cells were transfected with expression vectors as described under "Materials and Methods". After 4 h, tunicamycin (1 μ g/ml) was added (+) or not added (-) to the media. All the cells were harvested 28 h after transfection and analyzed by immunoblotting with anti-E2Tag antibody.

This pattern of products is similar to that of mammalian COX-1 and COX-2. The activity of recombinant caprellid COX was higher than that of the gammarid COX enzyme forming 2 μ g of products per a microsomal fraction of 1 million cells, while gammarid COX was able to form 1 μ g of products per 1 million cells. The activity of caprellid COX is similar to that of the coral *G. fruticosa* COX expressed in identical conditions (Järving et al., 2004).

We tested the effect of a nonselective mammalian COX inhibitor indomethacin and selective COX-2 inhibitor nimesulide on product formation by a recombinant amphipod COX. Neither indomethacin nor nimesulide up to concentrations of 100 μ M was able to inhibit the cyclooxygenase activity in the microsomal fractions of COS-7 cells transfected with the amphipod COX.

3.4. Structure of arthropod COX genes

The full-length gammarid and caprellid COX gene sequences were constructed from overlapping fragments obtained by PCR-cloning using gene-specific primers. In PCR reactions, the genomic DNA prepared from individual animals was used as a template. The sequences of intron/exon junctions were determined by comparing the cDNA and genomic sequences. In addition, the database searches of genomic sequences revealed the existence of two COX-like genes

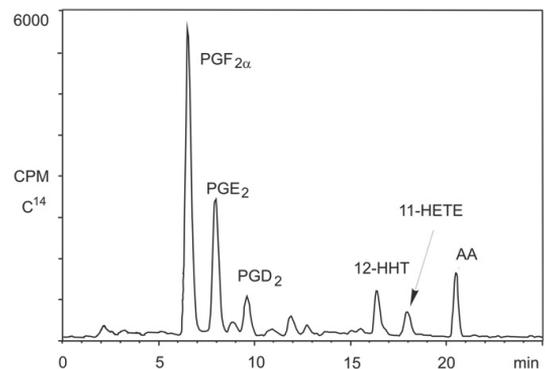


Fig. 5. RP-HPLC analysis of products formed by the caprellid COX expressed in COS-7 cells. [14 C]Arachidonic acid (50 μ M) was incubated with the microsomal fraction of COS-7 cells ($\sim 4 \times 10^6$) expressing the caprellid COX as described under "Materials and Methods". The products were analyzed using a Rigel ODS 5- μ m column (4.6 \times 250 mm) eluted at a flow rate of 1 ml/min with a stepwise gradient of the solvents: acetonitrile/0.017 M H $_3$ PO $_4$ (35/65, v/v) for 9 min, then acetonitrile/0.017 M H $_3$ PO $_4$ (70/30, v/v) for 9 min, and finally with acetonitrile. The peaks were detected using a UV detector coupled online with a liquid-scintillation counter. The radiolabelled products were identified by co-chromatography with added unlabeled standards. HHT, 12S-hydroxy-5Z,8E, 10E-heptadecatrienoic acid, AA, arachidonic acid.

in other arthropods, namely the human body louse *P. humanus corporis* (Insecta: Neoptera) (AAZ001002446) and the cladoceran crustacean *D. pulex* (Crustacea: Cladocera) (scaffold 9: 267142–269206).

The exon/intron borders in arthropod COX genes were compared with those of other reported invertebrate COX as well as human COX-1 and COX-2 genes. Maps of the compared COX gene structures are given in Fig. 6. The gammarid COX gene obtained is about 9.6 kb long and consists of thirteen exons and twelve introns. The caprellid COX gene is about 6.4 kb long and contains eleven exons and ten introns. The region of *D. pulex* COX gene corresponding to the open reading frame in mRNA comprises only five exons and four introns with a total length of about 2.0 kb. The exact length of the *P. humanus* COX gene is not determined due to the low sequence conservation in the region which corresponds to the N-terminal part of COX proteins. The exon–intron splice junctions all have the conserved GT–AG motifs. The intron splice sites and intron phases of gammarid and caprellid COX genes are shown in Table 2 (Supplementary material).

The structure of *P. humanus* and amphipod COX genes is similar to that of other known COX genes having six introns at conserved positions but only three of them are absolutely conserved in all arthropod and other COX genes reported (Fig. 6A). All introns at conserved positions are also in identical phases. In the *D. pulex* COX gene only introns that correspond to introns two, three, five and nine of the human COX-1 gene are present and the whole catalytic domain is separated between two long exons. The amphipod COX genes contain additional introns that are not found in any other COX gene (Fig. 6). The gammarid COX contains three additional introns and caprellid COX gene shares two of them. The length of introns in the amphipod COX genes varies markedly from 101 to 2500 bp in the gammarid gene and from 82 to 1500 bp in the caprellid gene (Fig. 6B,

Table 2 Supplementary material). The first introns of both genes are much longer than the others. All four introns in the *D. pulex* COX gene are with a similar length – from 73 to 88 bp. The introns identified in the *P. humanus* COX gene have a size from 78 to 145 bp.

4. Discussion

We have isolated genes from two crustacean amphipods that encode proteins corresponding to the mammalian COX. The major differences in primary structure between amphipod and mammalian COX lie in the N- and C-termini of proteins. The uncleaved signal sequence and the lack of the KDEL(STEL)-type ER retention/retrieval signal in the amphipod COX did not alter the post-translational processing of the protein in COS-7 cells and the recombinant enzymes were active. Our immunofluorescence and cell fractionating studies revealed that the subcellular localization of amphipod COX proteins on the ER and nuclear envelope was similar to that of mammalian cyclooxygenases. Mammalian COX-1 and COX-2 are integral membrane proteins associated monotonically with the luminal surfaces of the ER and on the inner and outer membranes of the nuclear envelope (Morita et al., 1995). Although several studies of mutation in both COX isoforms have shown that the N-terminal membrane binding domain rather than the C-terminal signal is responsible for the membrane binding and intracellular targeting, amino acid substitutions or deletions at the C-terminus in COX-1 generally led to a significant loss of catalytic activity (Ren et al., 1995; Li et al., 1998; Guo and Kulmacz, 2000). The longer C-terminal segment in COX-2 is distinctly more tolerant to structural changes than the shorter COX-1 C-terminal segment. Similar alterations to COX-2 had a markedly lower effect on activity (Guo and Kulmacz, 2000). The extremely short C-terminus of amphipod cyclooxygenases seems not to disturb the catalytic activity

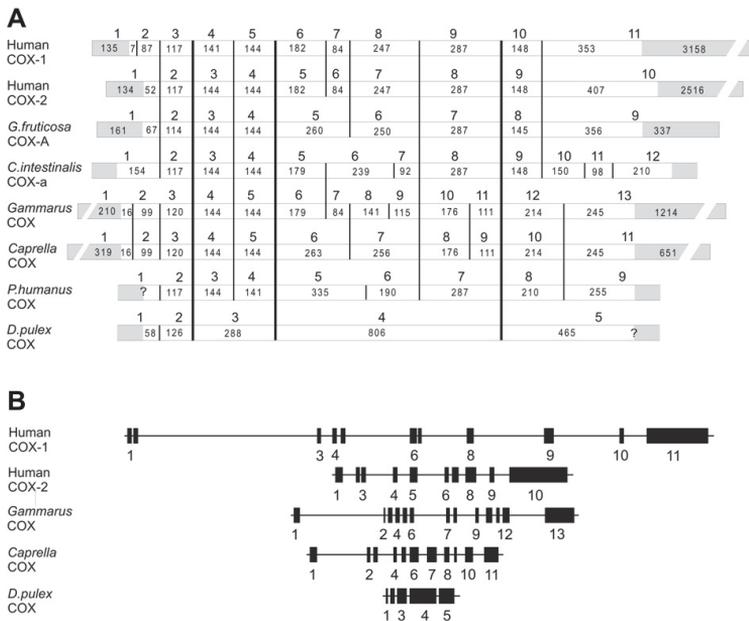


Fig. 6. Structure of human, coral, sea squirt, crustacean, and insect COX genes. (A) The number and length of the exons of gammarid COX, caprellid COX, *P. humanus* COX and *D. pulex* COX is compared with those of human COX-1 (GenBank accession nos. NM_000962 and AF440204) and COX-2 (GenBank accession nos. NM_000963 and D28235), *G. fruticosa* COX-A, and *C. intestinalis* COX-a genes (Järving et al., 2004). The numbers in the boxes indicate the numbers of nucleotides in each exon. The vertical bars mark intron/exon borders, bold bars indicate the absolute conservation of the site. The grey-filled areas identify the 5'- and 3'-untranslated regions. *The lengths of the first exon in the *P. humanus* COX gene and the fifth exon in the *D. pulex* COX gene are not exactly determined. (B) The exon/intron structure of human and arthropod COX genes, drawn to the same scale. The numbers identify the exons.

of enzymes. The ESTs representing partial COX sequences of two other malacostracan crustacean *Homarus americanus* (accession nos DV772953, EH401871) and *Petrolisthes cinctipes* (accession nos FE773225, FE699680) are available in the GenBank. The open reading frames of *H. americanus* and *P. cinctipes* cyclooxygenases have identical short C-termini and both proteins end with GTP sequences like the caprellid COX.

In vertebrate cyclooxygenases the potential sites for N-linked glycosylation are highly conserved. The vertebrate COX-1 is glycosylated at three asparagines. Site-directed mutagenesis studies have established that glycosylation at Asn410 is essential for enzyme activity (Otto et al., 1993). The vertebrate COX-2 is variably glycosylated at two to four asparagines, while Asn396 and Asn581 (human COX-2 numbering) are partially glycosylated (Nemeth et al., 2001). In invertebrate cyclooxygenases the glycosylation pattern is more heterogeneous: the gammarid and caprellid COX have four and three potential N-glycosylation sites, respectively, while the coral *G. fruticosa* COX-A has four, the *G. fruticosa* COX-B six, and the coral *P. homomalla* COX three potential N-glycosylation sites. Only one of the conserved N-glycosylation sites in vertebrate COX isozymes, at Asn144, is present in invertebrate cyclooxygenases but the location all the other sites in invertebrate enzymes is different. It has been shown that N-glycosylation is required for attaining a proper folding of the enzyme (Kulmacz et al., 2003). The present study and our previous works have shown that all these recombinant invertebrate cyclooxygenases are active in spite of essential differences in their glycosylation pattern (Koljak et al., 2001; Valmsen et al., 2001, 2004; Järving et al., 2004). This finding is indicative of the high variability of the N-glycosylation pattern depending on protein general structure.

The database search of the human body louse *P. humanus* genome revealed the existence of a COX-like gene, whose protein coding region is about 31–32% identical to that of mammalian COX-1 and COX-2, and shares a 34–35% sequence identity with that of amphipod cyclooxygenases. A comparison of intron positions and phases as well as exon sizes of the *P. humanus* COX gene with those of their mammalian orthologs is indicative of a high conservation,

thus suggesting a common evolutionary origin. However, several amino acids shown to be catalytically important like Arg120, Tyr385 and His207 are not conserved in the louse *P. humanus* COX.

The phylogenetic tree of cyclooxygenases and evolutionarily related heme proteins constructed by the neighbor-joining method forms two clearly distinct clusters; one is composed of vertebrate and invertebrate cyclooxygenases and the other includes peroxinectins (Pxt), myeloperoxidases (MPO) and peroxidasins (Pxd) (Fig. 7). This is in agreement with the results obtained by Zamocky et al. (2008) who aligned mammalian heme containing peroxidases together with similar peroxidase domain containing sequences from all kingdoms of life. It was shown that seven distinct subfamilies (including COX, Pxt, MPO, and Pxd) diverged from a common ancestor and retained common structural elements, but were segregated very early in evolution to perform different functions. The future isolation and characterization of these enzymes from insect species will explain their catalytic and biological functions within the insect world.

We have identified only one COX sequence in the human body louse *P. humanus* genome and in both the amphipod crustaceans investigated and one COX encoding gene was found in the cladoceran crustacean *D. pulex* genome (Heckmann et al., 2008). Mammalian species have two COX isoforms, the constitutively expressed COX-1 and inducible COX-2. The presence of two COX isoforms has also been established in birds and amphibians, but several fish species contain three COX genes (Järving et al., 2004; Ishikawa et al., 2007; Ishikawa and Herschman, 2007; Havird et al., 2008). Two COX genes have also identified in corals and sea squirts, but those genes have been emerged from gene duplication independently of the vertebrate COX-1 and COX-2 divergence (Järving et al., 2004). The phylogenetic tree indicate, while vertebrate COX sequences are strictly divided into COX-1 and COX-2 groups, the COX sequences of different classes of invertebrates form separate arms and show a considerable divergence, even though essential catalytic residues are conserved (Fig. 7).

In arthropods, the role of prostaglandins in reproduction and development, ion transport, fluid secretion, and immune defense

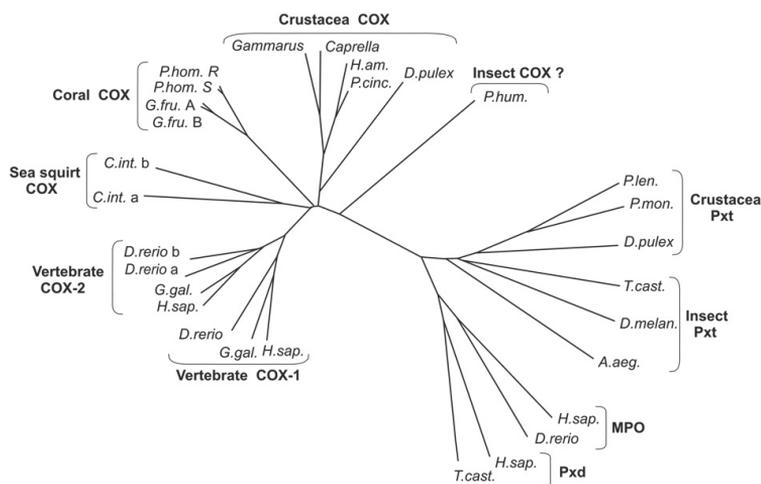


Fig. 7. Phylogenetic tree based on cyclooxygenases and related sequences from different vertebrates and invertebrates. The primary amino acid sequences from various organisms were obtained from the GenBank and Joint Genome Institute databases. Accession numbers for used sequences are listed in Table 3 (Supplementary material). Sequences were aligned using a multiple sequence alignment program ClustalW and the phylogenetic tree was constructed by the neighbor-joining method. *H.sap.*, *Homo sapiens*; *G.gal.*, *Gallus gallus* (chicken); *D.rer.*, *Danio rerio* (zebrafish); *C.int.*, *Ciona intestinalis*; *G.fru.*, *Gersemia fruticosa*; *Phom.*, *Plexaura homomalla*; *Ham.*, *Homarus americanus* (lobster); *P.cinc.*, *Petrolisthes cinctipes* (porcelain crab); *Phum.*, *Pediculus humanus corporis* (body louse); *Plen.*, *Pacifastacus leniusculus* (crayfish); *P.mon.*, *Penaeus monodon* (shrimp); *T.cast.*, *Tribolium castaneum* (beetle); *D.melan.*, *Drosophila melanogaster*; *A.aeg.*, *Aedes aegypti* (mosquito).

mechanisms has been reported by several authors (Stanley, 2000, 2006; Reddy et al., 2004; Tootle and Spradling, 2008; Machado et al., 2007). Whereas there was no evidence of the presence of the direct homologues of mammalian COX genes in completely sequenced insect genomes, it was proposed that insect enzymes involved in prostaglandin synthesis differ from their mammalian counterparts (Tootle and Spradling, 2008; Machado et al., 2007). At the same time, there are several studies describing the inhibition of biological processes in insects with mammalian COX inhibitors indomethacin and aspirin (Stanley, 2006; Tootle and Spradling, 2008; Machado et al., 2007). Surprisingly, our studies showed that the nonselective COX inhibitor indomethacin did not inhibit the activity of the recombinant amphipod COX.

Our results give clear evidence that prostaglandins in amphipod crustaceans are synthesized by the enzyme similar to mammalian COX. Crustaceans are believed to represent the ancestral arthropods from which insects have been originated (Glenner et al., 2006). It seems likely that at least some insect orders have lost the COX gene in the course of evolution. Future studies should illuminate which insect enzymes replace COX in oxylipin signaling.

Acknowledgements

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2009.10.002.

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Novel membrane-associated prostaglandin E synthase-2 from crustacean arthropods[☆]



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ABSTRACT

Prostaglandins (PG) have been shown to play important physiological roles in insects and marine invertebrates, yet the knowledge of their biosynthetic pathways is often lacking. Recently, we described cyclooxygenases in two amphipod crustaceans, *Gammarus* sp. and *Caprella* sp. In the present study, we report the cloning and characterization of prostaglandin E synthases (PGES) from the same organisms. The amphipod membrane-bound PGES-2-type enzymes share about 40% of the amino acid sequence identity with human mPGES-2, contain a conserved Cys110-x-x-Cys113 motif and have very low heme-binding affinity. The recombinant enzymes purified in the absence of dithiothreitol specifically catalyze the isomerization of PGH₂ into PGE₂. The PGES activity is increased in the presence of reduced glutathione and inhibited with a sulfhydryl group inhibitor. We assume that the amphipod mPGES-2, unlike in their mammalian counterparts, is responsible for PGE₂ synthesis, not only *in vitro* but also *in vivo*.

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1. Introduction

Prostaglandins (PGs) are well-known lipid mediators in vertebrates, and they have also been shown to play important regulatory roles in insects and other arthropods. In mammals, the biosynthesis of PGs occurs through multiple enzymatically regulated reactions. The process is initiated through the release of arachidonic acid (AA) from membrane phospholipids by the hydrolytic action of phospholipase A₂. The released AA is further metabolized into the unstable endoperoxide intermediate PGH₂ by the actions of PG endoperoxide synthase, also called cyclooxygenase (COX). Two distinct COX isozymes exist, COX-1 and COX-2, which are differently regulated. Once formed, the PGH₂ intermediate is converted to various prostanoids by specific PGH₂ isomerases and reductases (Smith et al., 2011).

Abbreviations: AA, arachidonic acid; GSH, glutathione; GST, glutathione S-transferase; COX, cyclooxygenase; HHT, 12(S)-hydroxy-5,8,10(Z,E,E)-heptadecatrienoic acid; HPLC, high performance liquid chromatography; MDA, malondialdehyde; Ni-NTA, nickel nitrilotriacetic acid; PGES, prostaglandin E synthase; cPGES, cytosolic prostaglandin E synthase; mPGES, membrane-associated PGE synthase; PG, prostaglandin; pHMB, p-Hydroxymercuribenzoate; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

[☆] Database linking: The mRNA sequences of amphipod mPGES-2 have been submitted to the GenBank database under the accession no. KC832830 (*Caprella* sp.) and no. KC832831 (*Gammarus* sp.), and the protein sequences under accession no.AGO64144 (*Caprella* sp.) and no.AGO64145 (*Gammarus* sp.).

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In mammals, prostaglandin E synthase (PGES, EC 5.3.99.3), which isomerizes COX-derived PGH₂ specifically to PGE₂, occurs in three structurally and biologically distinct forms (Kudo and Murakami, 2005). Cytosolic PGES (cPGES) is a glutathione (GSH)-dependent enzyme constitutively expressed in a wide variety of cells, and is functionally linked to COX-1 to promote immediate PGE₂ production (Tanioka et al., 2000). The two membrane-bound PGES enzymes have been designated as mPGES-1 and mPGES-2. mPGES-1 is a GSH-dependent perinuclear protein that is induced by proinflammatory stimuli, and that converts COX-2-derived PGH₂ to PGE₂ (Murakami et al., 2000). mPGES-2 is initially synthesized as a Golgi membrane-associated protein and the proteolytic removal of the N-terminal hydrophobic domain leads to the formation of a mature cytosolic enzyme (Watanabe et al., 1999; Tanikawa et al., 2002). This enzyme is constitutively expressed in various cells and tissues and is functionally coupled with both COX-1 and COX-2 (Murakami et al., 2003). Recently, it was reported that macaque mPGES-2 exists in two forms, as heme-free and heme-bound enzymes, that the heme-free enzyme catalyzes the formation of PGE₂ from PGH₂, and that the heme-bound mPGES-2 is a GSH-dependent protein which catalyzes PGH₂ degradation to 12(S)-hydroxy-5,8,10(Z,E,E)-heptadecatrienoic acid (HHT) and malondialdehyde (MDA). As the heme-free recombinant mPGES-2 converts to the heme-bound form if free heme is available, it was proposed that macaque mPGES-2 is a PGE₂ synthase *in vitro* but not *in vivo* (Takusagawa, 2013).

PGE₂ is the most common prostanoid in terrestrial and marine invertebrates and its physiological roles in reproduction, ion transport, immunity and defense reactions have been reported (Stanley, 2000;

Rowley et al., 2005; Stanley, 2011). The occurrence of PGE₂ has been shown in various marine arthropods, e.g. in the ovaries of the shrimp *Penaeus monodon* (Wimuttisuk et al., 2013), the kuruma prawn *Marsupenaeus japonicus* (Tahara and Yano, 2004), and the crab *Oziotelphusa senex senex* (Reddy et al., 2004), as well as in the previtellogenic ovary of the prawn *Macrobrachium rosenbergii* (Sagi et al., 1995). PGE₂ was identified in the secretory products of the parasitic copepod crustacean *Lepeophtheirus salmonis* (Fast et al., 2004) and in the blood cells of the shore crab *Carcinus maenas* (Hampson et al., 1992).

Although COX genes have been identified in all vertebrate animals investigated, there is little information about PG biosynthesis in lower animals and plants. In vitro biosynthesis of typical mammalian prostaglandins in invertebrates via the COX pathway was first reported in the soft coral *Gersemia fruticosa* (Varvas et al., 1994). To date, COX enzymes have been cloned and characterized in the soft corals *G. fruticosa* and *Plexaura homomalla* (Koljak et al., 2001; Valmsen et al., 2001; Valmsen et al., 2004) and in two amphipod crustaceans, *Gammarus* sp. and *Caprella* sp. (Varvas et al., 2009). COX has also been cloned and identified in the shrimp *P. monodon*, although the enzyme activity has not been examined (Wimuttisuk et al., 2013). In addition, the first non-animal COX was recently identified in the red alga *Gracilaria vermiculophylla* (Rhodophyta). The algal COX has only about 20% identity with human COX-1 and COX-2 and, unlike its mammalian counterparts, expresses easily in prokaryotic *Escherichia coli* cells as a highly active and fully functional enzyme (Kanamoto et al., 2011; Varvas et al., 2013).

There is also bioinformatic evidence available of a possible COX pathway in different invertebrates. Using genome database analysis, COX genes have been reported in the primitive chordates *Ciona savignyi* and *Ciona intestinalis* (Järving et al., 2004), in the crustaceans *Daphnia pulex*, *Homarus americanus*, and *Petrolisthes cinctipes* (Heckmann et al., 2008), and in the human body louse *Pediculus humanus corporis* (Varvas et al., 2009). However, homologs of mammalian COX genes have not been identified in completely sequenced insect genomes of *Drosophila* sp., *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Bombus mori*, *Tribolium castaneum* or others.

On the other hand, PGES-like sequences are common in arthropod genomes. While more than 30 predicted mPGES-2-like sequences have been identified in insects and other arthropod genomes so far, there is little information about the catalytic activity of corresponding proteins.

Here we describe the molecular cloning and characterization of functional mPGES-2 enzymes in the aquatic arthropod crustaceans *Gammarus* sp. and *Caprella* sp. Both enzymes specifically catalyzed the isomerization of PGH₂ (produced with algal COX) into PGE₂ *in vitro*. The protein and gene structures of amphipod mPGES-2 are briefly analyzed.

2. Materials and methods

2.1. Materials and reagents

[¹⁴C]AA was obtained from Perkin Elmer. The oligonucleotides were purchased from DNA Technology (Denmark). Restriction enzymes were obtained from MBI Fermentas. All other chemicals, if not mentioned otherwise, were obtained from Sigma-Aldrich. The crustacean samples were collected from the coast of the Kanagawa prefecture in Tokyo Bay and contained the red alga *G. vermiculophylla* and the small amphipod crustaceans *Gammarus* sp. and *Caprella* sp., which inhabit the macro algae community. The samples were stored at –80 °C until RNA isolation.

2.2. RNA isolation and cDNA cloning

Total RNA was extracted from tissue homogenate using SDS-guanidinium precipitation. The method is previously described by Su and Gabor (1988) and Koljak et al. (2001). mRNA was prepared from the total RNA using an oligo(dT)-cellulose column and purification kit

(Qiagen). The first strand cDNA was prepared using an oligo(dT)-adapter primer (Song et al., 1993).

Partial cDNA sequences coding amphipod mPGES-2 were obtained using nested PCR and two pairs of degenerative primers (Supplementary Table S1). 5'-RACE was accomplished using a 5'-RACE Kit (Roche Diagnostics). 3'-RACE was accomplished using the first strand cDNA prepared with an adapter-linked oligo(dT) primer. All the PCR products were cloned into the pGEM-T Easy vector (Promega), amplified in *E. coli* and sequenced.

cDNAs encoding the crustacean mPGES-2 proteins were amplified by PCR using the proofreading Phusion polymerase (Finnzymes). Primers carried BamHI restriction sites at their respective 5' ends, and upstream primers carried additional His₆-tags for the further purification of the recombinant proteins.

The amplified fragments were digested with BamHI, purified and cloned into the corresponding sites of the pET11-a vector (Novagen). The primers used for amplification of full-length and N-terminally truncated variants of gammarid and caprellid mPGES-2 are given in Supplementary Table S2.

2.3. Expression and purification of the recombinant enzymes

E. coli BL21(DE3)RP cells expressing the recombinant amphipod His₆-mPGES-2 were cultured in a 100 ml LB medium (containing 100 µg/ml ampicillin) alone or containing 0.2 mM FeCl₃ and 1.5 mM δ-aminolevulinic acid at 20 °C for 16 h following the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside at 0.6 of OD₆₀₀. The cells were harvested by centrifugation. All purification procedures were carried out at 4 °C. Recombinant proteins were purified with a nickel nitrilotriacetic acid (Ni-NTA) column, using a batch purification method (Qiagen). The cells were suspended with 3 ml of buffer A (30 mM potassium phosphate buffer, 1 M NaCl, 0.1 mM GSH, 1 mM phenylmethanesulfonyl fluoride and 0.01% octyl β-D-glucopyranoside, pH 8.0) and were disrupted by sonication for 5 × 5 s followed by centrifugation at 16000 × g for 10 min. After the addition of 20 mM imidazole, the supernatant was gently mixed with 0.5 ml Ni-NTA His-Bind slurry for 2 h. The protein-resin complex was packed into a column and washed with buffer B (30 mM potassium phosphate buffer, 1 M NaCl, 0.1 mM GSH, pH 8.0) containing 100 mM imidazole and 0.01% octyl β-D-glucopyranoside, and subsequently with buffer B (containing 100–135 mM imidazole). The enzyme was eluted with buffer B supplemented with 300 mM imidazole. The concentration of protein solution and the removal of imidazole were accomplished using continuous diafiltration through a MWCO 30 kDa filter (Pall Co.). Since the purified protein was relatively unstable and tended to aggregate easily, the filtration was carried out in the presence of 30 mM potassium phosphate buffer (pH 8.0), containing 1.5 M NaCl, 0.1 mM GSH, 30% glycerine and 0.03% octyl β-D-glucopyranoside (buffer C), in which protein aggregation was minimal.

The purity of the enzyme preparations was estimated by 15% SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined by SDS-PAGE, using a bovine serum albumin calibration curve, created according to protein band intensities and GeneTools software (Syngene). In a Western blot analysis, the proteins were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). His₆-mPGES-2 proteins were detected using a monoclonal anti-polyHistidine (mouse IgG2a isotype, Sigma).

2.4. Heme binding measurements

The heme content and heme binding to the purified amphipod protein were determined spectrophotometrically (Shimadzu UV-1601) at 240–600 nm. Heme binding to mPGES-2 was examined with titration analysis. 2.5 µl of freshly prepared 100 µM hemin in dimethyl sulfoxide was added to 0.5 ml of 8 µM purified protein solution in buffer C. The

heme concentrations ranged from 0.0 to 3.0 μM with increments of 0.5 μM .

2.5. PGE synthase activity assay

The catalytic activity of mPGES-2 was measured, using a coupled enzyme assay (Meyer and Thomas, 1995; Meyer et al., 1996; Thomson et al., 1998). To ensure continuous synthesis of fresh PGH_2 , algal (*G. vermiculophylla*) COX enzyme was used (Varvas et al., 2013).

Reactions were carried out in 0.5 ml of 0.1 M potassium phosphate buffer (pH 8.0) containing 1 mM GSH, 2 μg purified algal COX and various amounts (50–100 μg) of purified N-terminally truncated recombinant mPGES-2 or the crude cell extract. After preincubation for 3 min, [^{14}C]AA (final concentration of 50 μM) was added, and incubation was carried out for 3 min at room temperature. The reactions were terminated by the addition of 10 mM SnCl_2 and the products were extracted and analyzed by thin-layer chromatography or RP-HPLC, as described previously (Varvas et al., 1999; Järving et al., 2004). In inhibition studies, the algal COX and amphipod mPGES-2 were preincubated for 3 min at room temperature in the presence of 5 mM sodium salt of p-hydroxymercuribenzoate (pHMB) and the reaction was carried out under the conditions described above.

2.6. Isolation, amplification and cloning of genomic DNA

Genomic DNA was isolated using the cetyltrimethylammonium bromide procedure described previously (Varvas et al., 2009). The DNA fragments were amplified by PCR with Taq DNA polymerase, and the genomic DNA prepared from individual animals was used as a template. The gene specific primers were constructed using cDNA data (Supplementary Table S2). The PCR products were cloned into the pGEM-T Easy vector, amplified in *E. coli* and sequenced. The exon/intron boundaries were determined by a comparison of genomic DNA fragments with full-length cDNA sequences.

2.7. Bioinformatic and sequence analysis

The cloned PCR fragments were sequenced by LGC Genomics (Berlin, Germany). Sequence analysis was performed using Lasergene programs (DNASTar, Inc.). Protein sequences of related PGES were found by BLASTP 2.2.26+ and domains were identified using the conserved domain database (Marchler-Bauer et al., 2011). Tertiary structure models of amphipod mPGES-2 were constructed using CPHmodels 3.0 (Nielsen et al., 2010) on the basis of *Macaca fascicularis* mPGES-2 crystal structure (PDB: 2PBJ), and analyzed with Chimera 1.6.2. The subcellular localization of recombinant amphipod mPGES-2 proteins was predicted by TargetP and PSORT programs. A topological study was performed with the program TopPred 0.01. The web addresses for the programs are listed in Supplementary Table S4.

3. Results

3.1. cDNA cloning and sequence analysis

The cDNA sequences encoding mPGES-2 were cloned from the amphipod crustaceans *Gammarus* sp. and *Caprella* sp. using a RT-PCR strategy. mPGES-2 coding open reading frames were 1293 bp for both amphipods (430 amino acids). *Gammarid* mPGES-2 mRNA contained a 68 bp 5'-untranslated region (UTR) and a 41 bp 3'UTR. The *Caprellid* mPGES-2 mRNA sequence was comprised of a 41 bp 5'UTR and a 77 bp 3'UTR. The predicted molecular mass of the amphipod full-length proteins was 50 kDa and the proteins shared 57% identity in their amino acid sequences.

The mammalian mPGES-2 is comprised of an N-terminal membrane-associated region and a cytoplasmic glutathione S-transferase (cGST)-like region, which included a thioredoxin-like domain, and a C-terminal helical domain (Daiyasu et al., 2008). The thioredoxin-like domain of mPGES-2 had a consensus thioredoxin homology sequence of Cys110-x-x-Cys113, shown to be responsible for catalytic activity (Watanabe

1	MI_VL_SRYFLNLSI_SRFSS--TCLLPKRCL_SRL_VL_DAOAKGQPSRRPFL_HSCRCYAQKAKAAEI_KEMPADKRKMKYI_VMGAVGF_GAYI_VA	Gammarid mPGES-2
1	MNSVSKFEVFNWGRCL_KL_SSRSHVAPLYSL_SLDKQQL_SRTFSNSTFEFSQSRSQSSQSKSDANI_KSFTFS--RNTKL_TVGL_GLGL_GAYLLA	Caprellid mPGES-2
1	-----MNHKPSKFLG-----R-----ISSAYSPSPPLASTSPRCYASSSSPKLSTSP-----RLALGASVAGGL_YAL	L. salmonis mPGES-2
1	-----MSCI_RL_ASTVALAGVRPPAAGGICAL_RLL_TKRNPDAFAARRQF_AVAENGGRKKPNGT_FK_LAVL_GAVT_VGAA_TGSVYT	D. melanogaster Su(P)
1	-----MDPAARVVRL_WP_GGICAL_AWRL_GGRPOPL_LPTQSRAGFAAGAGGQGPVA_AARKGSPRL_L_GAAAL_LGAL_GL	H. sapiens mPGES-2
1	-----MAPATRVRL_WI_GGICAL_AWRL_GGRPOPL_LPTQSRAGFAAGAGGQGPVA_AARKGSPRL_L_GAAAL_LGAL_GL	M. fascicularis mPGES-2
	Y107 C110 C113	
89	LL_FYEKAAL_DERLKEERRAKSL_TMQPLPYL_Q_EPPPTPAI_ARSVYNPNDSGL_KL_TLYQYQTC_PFCCKVRAFL_DYYGF_SVDVVEVNSI_TR	Gammarid mPGES-2
90	L_DQWNGSKEN-----ERTAMMLRTDGLPYI_D-DKPPENVARKI_FNPKDQTGI_KL_TLYQYQTC_PFCCKVRAFL_DYYGF_SVDVVEVNSI_TR	Caprellid mPGES-2
90	YNFWPTQSQSS-----QI_VQMPNDESEI_PHI_LPEAPPNDKCI_KI_RYPEDKTKL_TLYQYQTC_PFCCKVRAFL_DYYGF_SVDI_VEVNSI_FR	L. salmonis mPGES-2
80	MYQRWFDGSSSHEHEET_KPTRLDGI_PAGVRI-----TKRYVNPKDT_SGLDI_VL_FQYQTC_PFCCKVRAFL_DYMG_I_SVAVVEVDVLR	D. melanogaster Su(P)
73	YHTARWHL_RAQDL_HAERSAAQL_S-----LSSRL_QL_TLYQYQTC_PFCCKVRAFL_DFHAL_PYQVVEVNPVRR	H. sapiens mPGES-2
73	YHTARWHL_HAQDL_HAERSAVQL_S-----LSSRL_QL_TLYQYQTC_PFCCKVRAFL_DFHAL_PYQVVEVNPVLR	M. fascicularis mPGES-2
	P149	
178	KDI_KVS_EYKVPAL_VVEVPG--DKKSTFI_QL_KDSNVII_SLLSSCLRNPGESLDTLLASYPVYSDAE--NDNKLFQDNRYFI_MYTEI_EG	Gammarid mPGES-2
174	KDVKWS_SYKVPFLI_VESPGSTDGETKYI_QL_KDSNVII_SLLGSCLRNPESLHKLLEESFPSYDGD--NGNKLQFANRYFI_MYTETKD	Caprellid mPGES-2
146	KEI_KWSPNYKVP_LVVKTPQ----GDFLQL_NDSTMII_SALMTYLKEPSKNL_KDI_VECYPQVNTDPSSGKSTI_EI_RNKYFI_MHQNDS-	L. salmonis mPGES-2
161	QDI_RWS_SVKVPMWLI_RQDDGK----YVQWDSAI_SLI_ATHLQDKRTDI_GELAQFYPHTSFFD-DGKKNKDI_LNKYFL_MYREHTP	D. melanogaster Su(P)
138	AEI_KFS_SYRKVPI_LVAQEGES----SQQL_NDSSVII_SALKTYLVS_GQPLEEII_TYYPAMKAVN-EQKVEVTEFNKYYW_ML_NEKEA	H. sapiens mPGES-2
138	AEI_KFS_SYRKVPI_LVAQEGES----SQQL_NDSSVII_SALKTYLVS_GQPLEEII_TYYPAMKAVN-DQKVEVTEFNKYYW_ML_NEKEA	M. fascicularis mPGES-2
	H244 S247 Y251 G268 R292 R296	
262	LPKK--QKYI_KRERRL_RRWDDKFVHTI_SPNVFRTPSEAL_QAFHWVSOAEGWDFVSSVERLL_VVYAGAAMMMI_GKLLKKRYNI_SPDPR	Gammarid mPGES-2
260	LERK--QKYI_KLERKRWQWDDKFVHTL_SPNVYRTPSEL_QAFNWFDEVGDWQRL_FSSVERFLVI_YL_GASVMMI_LGKRLKKRHNI_TSDPR	Caprellid mPGES-2
230	--VN--DKNLKEEL_KWRKWDSFVFRHISPNYRSLSESI_EAFHWDTAGKKNVNTFFERQVVI_YL_GASVMMI_LGKVLKKRHLPDDVR	L. salmonis mPGES-2
244	KGVS--KETDED_RKWRVWADSHLVHLI_SPNCYQTMGESLTFEWFVSOAEGWDFVHPKVERDL_MVYCGATAMMYI_AKMLKRRHAL_SDDVR	D. melanogaster Su(P)
219	QQVYGGKEART_EEMKRWQAWDDL_VHLI_SPNVYRTPTEALASFYI_VREGK----FGAVEGAVAKYMGAAAMMYI_SKRLKSRHRL_QDNVR	H. sapiens mPGES-2
219	QQVYSGKEART_EEMKRWQAWDDL_VHLI_SPNVYRTPTEALASFYI_VREGK----FGAVEGAVAKYMGAAAMMYI_SKRLKSRHRL_QDNVR	M. fascicularis mPGES-2
	*K269	
350	L_TLYDQADL_WRELNEED--FVGGENPNLADL_GMYGALS_SMEGCKFAEMLEKTKI_GSWYDKMKGRI_QERRGEYKLVRSI_I_T	Gammarid mPGES-2
348	I_SL_YDQANL_WMRI_RTSEGPFGMGTTPNLADL_SMYGVS_SAI_EGDAFDQDL_DNNTKI_GKWFYKMKVVLAREGEEKLVKRCISI_R	Caprellid mPGES-2
316	EAI_YLNNYLSHWYKKGGSFGGVEFDLADI_SFYGTLSNFECAAFQDVL_SHTLE_RQWV_TKMKVAVSSRAGSSI	L. salmonis mPGES-2
332	SHMYDAL_DQWTEL_KRNTKFMGGKQPSLADL_SVFG_SSMGECOT_KDCLQNTSI_GKWFYDVKAL_VKRNQGLQRERI_ENMAAI_A	D. melanogaster Su(P)
305	EDL_YEADKWAAVGK--DRPFMGGQKPNLADLAVY_GVLRVMEGLDAFDDL_MQHTHI_QPWLVVERAI_TEASPAH	H. sapiens mPGES-2
305	EDL_YEADKWAAVGK--DRPFMGGQKPNLADLAVY_GVLRVMEGLDAFDDL_MQHTHI_QPWLVVERAI_TEASPAH	M. fascicularis mPGES-2

Fig. 1. Multiple alignment of deduced amino acid sequences of the mPGES-2 from *Gammarus* sp. and *Caprella* sp. amphipods, sea louse (*L. salmonis*), human (*H. sapiens*), macaque (*M. fascicularis*) and the Su(P) protein of the fruit fly (*D. melanogaster*). Identical amino acid residues are shaded in gray. The N-terminal hydrophobic domain (determined for human and macaque, and predicted for gammarid and caprellid mPGES-2) which is cleaved proteolytically is underlined with a solid line. Catalytically important amino acids are indicated with asterisks and numbers. The Cys-Pro-Phe-Cys motif and predicted GSH-binding motif are boxed. Accession and database sequence identifiers are given in Supplementary Table S3.

et al., 2003). The conservation of the membrane-associated N-terminal hydrophobic domain and the C-terminal cytosolic domain with the cGST-like structure in the amphipod PGES sequences was confirmed by TopPred 0.01 and the conserved domain database (Marchler-Bauer et al., 2011). Both amphipod enzymes contained a glutaredoxin/thioredoxin homology region, which included the catalytically important Cys–Pro–Phe–Cys motif. The predicted GSH-binding motif Val148–Pro–x–Leu...Asp164–Ser–x–x–Ile (Takusagawa, 2013) was also conserved in amphipod proteins (Fig. 1). The subcellular localization of amphipod mPGES-2 was predicted to be mitochondrial by both TargetP and PSORT programs.

The invertebrate mPGES-2-like sequences had a longer N-terminal region than that of mammalian mPGES-2 (Campbell et al., 2009), yet amphipod mPGES-2 had an N-terminal part which was the longest among known mPGES-2-sequences. mPGES-2 from *Gammarus* sp. and *Caprella* sp. share 40–43% amino acid sequence homology with human mPGES-2. The highest (48–51%) sequence conservation was found with PGES-2-like sequences of the prawn *P. monodon*, sea louse *Caligus rogercresseyi* and water flea *D. pulex*.

The predicted protein structure model of caprellid mPGES-2 was compared with an *M. fascicularis* mPGES-2 crystal structure (PDB: 2PBJ). The residues 1–99 that were part of the truncated N-terminal section and disorder section in the macaque's mPGES-2 were not included in the crystal structure, and the amphipod mPGES-2 structure was also proportionately truncated (135 residues). The superimposed structures, sharing 43.7% of identical residues, showed remarkable similarity. The RMSD between 233 α -carbon atom pairs (79% of total) was 0.840 Å (Fig. 2).

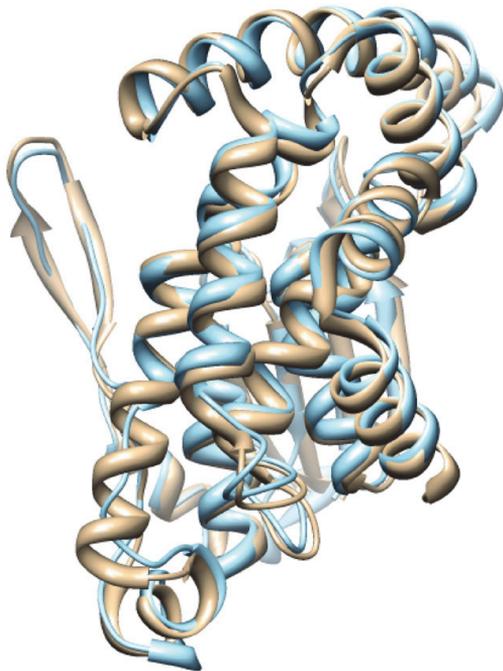


Fig. 2. A superimposed view of the *Macaca fascicularis* mPGES-2 crystal structure and the predicted three-dimensional structure of *Caprella* sp. mPGES-2. The view was constructed using a *Macaca fascicularis* mPGES-2 monomer. Gray indicates macaque, and blue caprellid mPGES-2 enzyme structure. Residues 1–99, which are part of the truncated N-terminal section and disorder section, are not shown in the macaque's mPGES-2 crystal structure. The amphipod mPGES-2 modeled structure is also proportionately lacking 135 residues.

3.2. Expression and purification of amphipod recombinant mPGES-2

Mammalian mPGES-2 is a membrane-anchored dimeric protein (Yamada et al., 2005) and the proteolytic removal of the N-terminal hydrophobic domain (87 amino acids) leads to the formation of a mature cytosolic protein. It has been shown that both the full-length and the N-terminally truncated mPGES-2 overexpressed in *E. coli* have the same catalytic properties (Tanikawa et al., 2002). Commonly, the soluble N-terminally-truncated mPGES-2 is expressed and examined. Therefore, according to multiple sequence alignment of different mPGES-2 proteins and appropriate amino acid organization, the amphipod enzymes were N-terminally truncated at 103 and 99 amino acid residues for gammarid and caprellid mPGES-2, respectively. To facilitate purification by affinity chromatography, a His₆-tag was attached to the truncated N-terminus.

The amphipod mPGES-2 enzymes were functionally expressed in *E. coli* cells at 20 °C for 16 h. The recombinant proteins were purified by nickel affinity chromatography, in the presence of GSH. The addition of a detergent octyl β -D-glucopyranoside to the washing solutions increased the purity of the proteins. A sample from each purification step was subjected to SDS-PAGE (Fig. 3A). Western blot analysis revealed that the dominant band was recognized by the anti-His antibody (Fig. 3B). The amphipod mPGES-2 enzymes were purified to apparent homogeneity as determined by SDS-PAGE, where only traces of other proteins could be detected (Fig. 3). On average, 5–7 mg of purified protein was obtained from 1 l of bacterial culture. The molecular mass for gammarid and caprellid N-terminally truncated His₆-mPGES-2 was equally 39 kDa, as determined by SDS-PAGE.

3.3. Heme content and heme binding to amphipod mPGES-2

The heme content of the purified mPGES-2 was measured spectrophotometrically. According to the absorption spectrum (Fig. 4), the

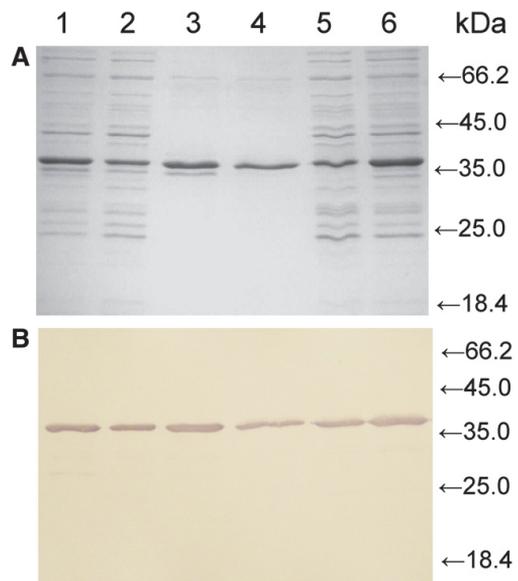


Fig. 3. Purification of amphipod recombinant mPGES-2 enzymes. (A) SDS-PAGE (2 μ g of protein each line) and (B) Western blot analysis of His₆-mPGES-2 proteins at different stages of purification. Lanes 1–3 represent caprellid His₆-mPGES-2 and 4–6 correspond to gammarid His₆-mPGES-2. Crude cell extract (lanes 1 and 6); 16000 \times g supernatant (lanes 2 and 5); after Ni-NTA column (lanes 3 and 4).

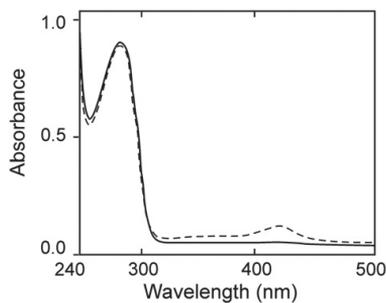


Fig. 4. Absorption spectra of purified mPGES-2 enzymes. The solid line represents the enzyme expressed in LB medium alone and the dotted line represents the mPGES-2 from LB medium containing 0.2 mM FeCl₃ and 1.5 mM δ -aminolevulinatate. The purified mPGES-2 enzymes contained 300 mM imidazole and 1.0 mM GSH, thus causing the shift of the heme absorption spectrum to 418 nm.

recombinant enzyme expressed in LB medium alone did not contain any heme. The enzyme expressed in the presence of 0.2 mM FeCl₃ and 1.5 mM δ -aminolevulinatate showed very weak heme-binding capacity (the heme and protein peak height ratio A_{418}/A_{280} was 0.062). These results were not consistent with the recombinant mammalian mPGES-2 enzyme, where heme content in each protein subunit was 78% (expression in LB medium alone) and 100% (expression in LB medium containing 0.25 mM Fe(NO₃)₃ and 1.5 mM δ -aminolevulinatate), respectively (Takusagawa, 2013). In addition, different from mammalian enzyme, purified amphipod mPGES-2 did not bind heme in the presence of GSH. Titration of the purified mPGES-2 with heme solution in the presence of 0.1 mM GSH revealed no change in the UV–visible absorbance spectrum of heme (Fig. 5). The position and shape of the heme peak in protein solution were similar to free heme in buffer solution (in the

absence of the enzyme), indicating that heme does not have a specific interaction with amphipod mPGES-2.

3.4. Enzymatic activity of amphipod mPGES-2 enzymes

Since PGH₂ is extremely unstable in aqueous solution in vitro and is spontaneously and non-enzymatically converted to a mixture of PGE₂, PGD₂, and PGF_{2 α} (Yu et al., 2011), the catalytic activity of amphipod mPGES-2 was studied in a “one-pot process”, in which PGH₂ was both generated and metabolized without isolating the intermediate. The substrate for mPGES-2, PGH₂, was synthesized from [¹⁴C]AA, using recombinant COX from the red alga *G. vermiculophylla*.

To exclude the possibility that the bound heme would dissociate from the enzyme during purification procedures and alter its catalytic activity, both the crude cell extract and purified recombinant mPGES-2 were used in activity assays. Both enzyme preparations converted algal COX-derived PGH₂ specifically to PGE₂. No significant conversion of PGH₂ to HHT and MDA was detected. Similar results were obtained for the enzymes expressed in the LB medium alone or in the presence of 0.2 mM FeCl₃ and 1.5 mM δ -aminolevulinatate. Also, the addition of heme to the assay mixture did not initiate the degradation of PGH₂ to HHT and MDA.

Control experiments were performed without the addition of amphipod mPGES-2 in the reaction mixture (Fig. 6 and Table 1). Due to the instability of the PGH₂ synthesized by algal COX in the aqueous medium, it was not possible to quantify the specific PGE synthase activity; however, the results clearly showed that amphipod mPGES-2 specifically promoted PGE₂ formation. The small amounts of PGD₂ and PGF_{2 α} observed can only be explained by the rapid degradation of the highly unstable PGH₂ in the aqueous medium.

During purification of the amphipod mPGES-2, about 80% of their initial activity was lost. The instability of the amphipod enzymes was not surprising, as in the heme-free state the mammalian enzyme has

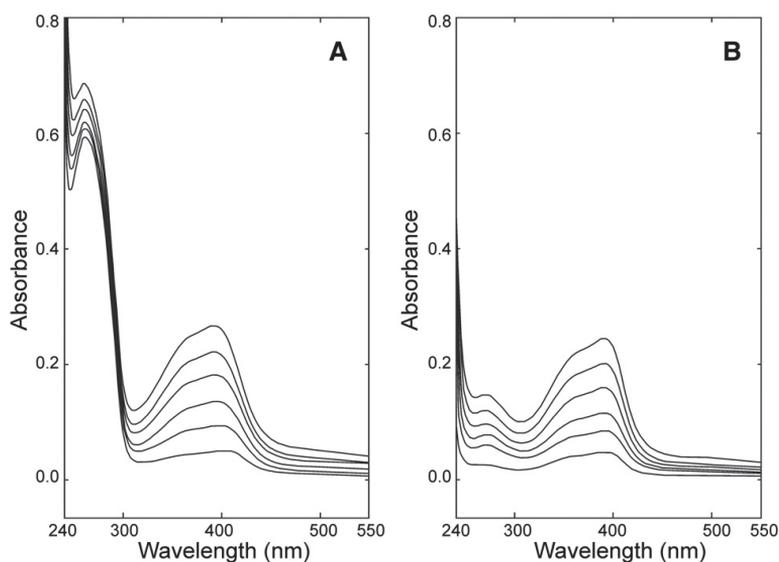


Fig. 5. Absorption spectra of purified mPGES-2 upon sequential titration with heme. The mPGES-2 concentration was 8 μ M and the heme concentration was raised from 0.0 to 3.0 μ M, with increments of 0.5 μ M. The titration was carried out in the presence of 0.1 mM GSH. (A) *Caprella* sp. mPGES-2. (B) Buffer C in the absence of the enzyme.

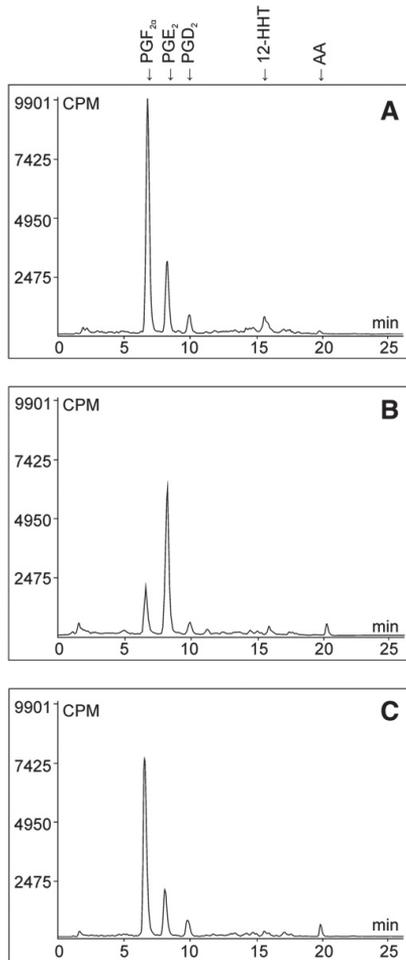


Fig. 6. PGE synthase activity of caprellid mPGES-2. The PGE synthase activity was measured in a coupled assay incubating algal COX with [14 C]AA as a substrate in the absence (A) or the presence (B) of 100 μ g purified recombinant caprellid mPGES-2. In (C) the coupled reaction mixture was preincubated with a SH-group inhibitor pHMB. Individual assays, typical of several similar experiments are shown.

also been shown to be very unstable: only 20% of the initial PGE synthase activity was recovered when the human heme-free mPGES-2 was purified from *E. coli* cells (Tanikawa et al., 2002).

Table 1
PGE synthase activity of recombinant amphipod mPGES-2. The catalytic activity of mPGES-2 was measured, using a coupled enzyme assay with algal COX as described in the Materials and methods section. The radio-labeled products were extracted and analyzed by TLC. Plus indicates the presence and minus the lack of the corresponding enzyme or inhibitor in the reaction mixture. The results are expressed as means \pm S.D. for four determinations. Values are statistically different as determined by Student's *t* test ($p < 0.005$).

Algal COX 2 μ g	Caprellid mPGES-2 100 μ g	Gammarid mPGES-2 100 μ g	Inhibitor pHMB 5 mM	Ratio of PGE ₂ /PGE _{2x}
+	–	–	–	0.3 \pm 0.1
+	+	–	–	3.5 \pm 0.5
+	–	+	–	2.4 \pm 0.4
+	+	–	+	0.3 \pm 0.1

Our results indicate that GSH increased both the stability and activity of amphipod mPGES-2. The activity of the enzyme was augmented 4 to 5-fold in the presence of 0.1–1.0 mM GSH. When 5 mM sodium salt of the well-known SH-group inhibitor pHMB was added to the reaction mixture, the mPGES-2 activity was completely inhibited (Fig. 6C and Table 1), confirming the catalytic importance of the Cys110-x-x-Cys113 motif.

3.5. Structure of amphipod mPGES-2 genes

The full-length sequences of gammarid and caprellid mPGES-2 genes were constructed from overlapping fragments obtained by PCR-cloning using gene-specific primers. The structural organization and the exon-intron boundaries of amphipod mPGES-2 genes were determined by comparing the cDNA and genomic sequences. mPGES-2 genes from caprellid and gammarid spanned about 4.1 kb and 4.3 kb of DNA, respectively. Both genes consisted of 7 exons. The exon-intron boundaries of amphipod mPGES-2 genes were compared with those of other reported invertebrate mPGES-2, as well as human mPGES-2 genes (Fig. 7). All splice acceptor and donor sequences followed the AG-GT consensus rule. The exon lengths of mPGES-2 genes were highly conserved in all mammalian species examined, but varied among invertebrates, which tend to have longer exons and smaller number of introns. Due to their longer N-terminal domain, both amphipod mPGES-2 genes contain one additional exon not found in other organisms. Only intron four of the human mPGES-2 gene is conserved in all studied invertebrate mPGES-2-like genes. The length of introns in the amphipod mPGES-2 genes varied from 150 to 900 bp, being significantly longer than in other arthropod mPGES-2 genes. All introns at conserved positions were in identical phases.

4. Discussion

PGES-like sequences have been found in many arthropods. Bioinformatic studies indicate that mPGES-2-type PGE synthase is the most widespread among arthropods, although in some cases the cPGES and mPGES-1 types are also represented, but all three PGES sequences have been described only in the peaneid shrimp *P. monodon* (Wimuttisuk et al., 2013).

The phylogenetic analysis revealed that arthropod mPGES-2 sequences formed a clearly distinct cluster compared to vertebrate enzymes (Fig. 8); the same effect was shown for PGES-1 sequences (Wimuttisuk et al., 2013). The analysis supports the fact that arthropods segregated very early in evolution and developed independently.

Recently, Takusagawa reported that mammalian mPGES-2 is a GSH-dependent heme protein and *in vitro* dithiothreitol dissociates the bound heme to produce active heme-free PGE₂ synthase (Takusagawa, 2013). To avoid heme dissociation, amphipod mPGES-2 enzymes were purified in the absence of dithiothreitol and in the presence of GSH. Our results show that mPGES-2 enzymes in aquatic arthropods have significantly lower heme binding affinity than mammalian mPGES-2. Therefore, they may exist *in vivo* as heme-free proteins capable of catalyzing the synthesis of PGE₂.

To date, there is little information about the function of PGES-2-like enzymes in arthropods. In this study, we clearly demonstrated that amphipods use the prostaglandin synthesis pathway, where PGE₂ is formed from arachidonic acid via successive reactions of COX and mPGES-2. So far, the function of PGES-like enzymes in organisms lacking COX and its substrate fatty acids is not known. These enzymes could carry out some other physiologically important function(s). For example, the mPGES-2 homolog in *Drosophila melanogaster*—Su(P) protein has been shown to play a role in male fly fertility (Bichon et al., 2001) and *L. salmonis* has a ubiquitous and constitutively expressed mPGES-2 enzyme which seems to be more like a detoxifying GST than the enzyme involved in PGE₂ synthesis (Campbell et al., 2009).

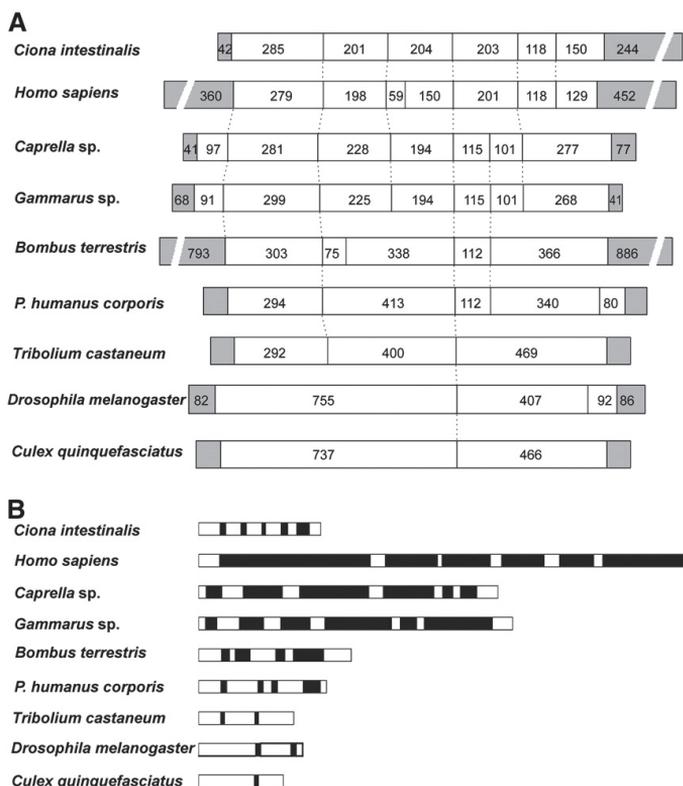


Fig. 7. Comparison of mPGES-2 gene structures from human (*Homo sapiens*) and different arthropods and the *Su(P)* gene from the fruit fly (*Drosophila melanogaster*). (A) The numbers in the boxes indicate the numbers of nucleotides in each exon. The vertical dotted lines indicate conservation of the intron positions. The gray-filled areas identify the 5'- and 3'-untranslated regions. (B) The exon–intron structures of mPGES-2 genes from different organisms are presented in the same scale. Exons are shown with open and introns with filled boxes. Accession and database sequence identifiers are given in Supplementary Table S3.

Still, the exact role of mPGES-2-like enzymes in terrestrial insects and other invertebrates remains to be determined.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbpb.2014.05.004>.

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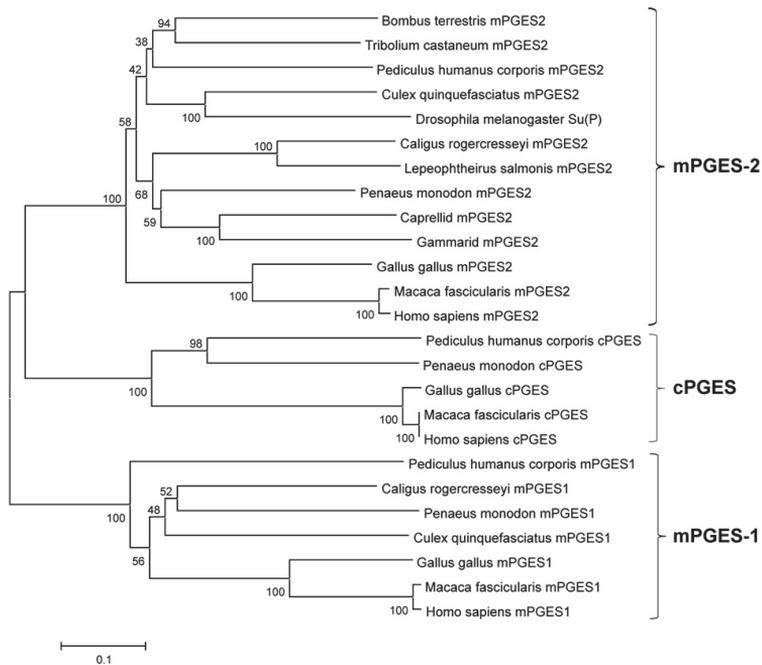


Fig. 8. Phylogenetic tree of the PGES family. The tree was constructed with MEGA 5.10 using amino acid sequences with the neighbor-joining method. The scale bar represents an estimate of the number of amino acid substitutions per site. Bootstrap values are indicated for each branch divergence. Accession and database sequence identifiers are given in Supplementary Table S3.

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CURRICULUM VITAE

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Education

Educational institution	Graduation year	Education (field of study/degree)
Tallinn University of Technology	2007	Applied Chemistry and Biotechnology, master's degree
Tallinn University of Technology	2005	Gene Technology, bachelor's degree
Elva Gymnasium	2002	

Language competence/skills (fluent, average, basic skills)

Language	Level
Estonian	Fluent
English	Average

Professional employment

Period	Organisation	Position
Since 2008	Tallinn University of Technology, Department of Chemistry	Research scientist

Thesis supervised:

Enelin Pihlak „Prostaglandin E synthase-2 in corals *Gersemia fruticosa* and *Capnella* sp." Tallinn University of Technology, BSc, 2012.

Research activity:

Bioorganic chemistry. Protein expression and purification, structure and function analysis.

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Hariduskäik

Õppeasutus (nimetus lõpetamise ajal)	Lõpetamise aeg	Haridus (eriala/kraad)
Tallinna Tehnikaülikool	2007	Rakenduskeemia ja biotehnoloogia, magistri kraad
Tallinna Tehnikaülikool	2005	Geenitehnoloogia, bakalaureuse kraad
Elva Gümnaasium	2002	Keskharidus

Keelteoskus (alg-, kesk- või kõrgtase)

Keel	Tase
Eesti	Kõrgtase
Inglise	Keskase

Teenistuskäik

Töötamise aeg	Tööandja nimetus	Ametikoht
Alates 2008 a.	TTÜ, Keemiainstituut	teadur

Juhendatud lõputööd:

Enelin Pihlak „Prostaglandiin E süntaas-2 korallides *Gersemia fruticosa* ja *Capnella* sp.“ Tallinna Tehnikaülikool, bakalareusetöö, 2012.

Teadustegevus:

Bioorgaaniline keemia. Valkude ekspressioon ja puhastamine, struktuuri ja funktsioonianalüüsid.

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