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Structural and functional comparison of 15*S*- and 15*R*-specific cyclooxygenases from the coral *Plexaura homomalla*

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It has been known for 30 years that the gorgonian coral *Plexaura homomalla* contains either 15*S*- or 15*R*-configuration prostaglandins (PGs), depending on its location in the Caribbean. Recently we showed that the 15*R*-PGs in the *R*-variety of *P. homomalla* are formed by a unique cyclooxygenase (COX) with 15*R* oxygenation specificity [Valmsen, K., Järving, I., Boeglin, W.E., Varvas, K., Koljak, R., Pehk, T., Brash, A.R. & Samel, N. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 7700]. Here we describe the cloning and characterization of a closely related COX protein (97% amino acid sequence identity) from the *S*-variety of *P. homomalla*. Functional expression of the *S*-variant COX cDNA in Sf9 insect cells followed by incubation with exogenous arachidonic acid resulted in formation of PG products with > 98% 15*S*-configuration. Mutational analysis was performed on a suggested active site determinant of C-15 oxygenation specificity, position 349 (Val in all *S*-specific COX, Ile in 15*R*-COX). The 15*S*-COX Val349 to Ile mutant formed 35% 15*R*-PGs, while the reverse mutation in the 15*R*-COX (Ile349Val) led to formation of 70% 15*S*-products. This establishes position 349 as an important determinant of the product stereochemistry at C-15. Our characterization of the enzyme variants demonstrates that very minor sequence divergence accounts for the content of epimeric PGs in the two variants of *P. homomalla* and that the differences do not arise by isomerization of the products.

Keywords: cyclooxygenase; *Plexaura homomalla*; 15*R*-prostaglandins; site directed mutagenesis; stereospecificity.

Cyclooxygenase (COX) enzymes catalyse the conversion of arachidonic acid to prostaglandin (PG) endoperoxide, the precursor of PGs and thromboxanes [1–5]. PG hormones act as important mediators in tissue homeostasis and also in inflammation and cancer [6–10]. Synthesis of PGs involves an initial oxygenation at C-11 of arachidonic acid, followed by two cyclization reactions and a final reaction with molecular oxygen at C-15. In vertebrates, the *S*-configuration of the carbon-15 is crucial for the biological activity of PGs [11,12], and therefore the COX enzyme strictly controls the stereochemistry of the reaction with molecular oxygen, resulting exclusively in formation of 15*S*-products.

When PGs were first discovered in marine life in the Caribbean coral *Plexaura homomalla* collected from the Florida Keys, it turned out that the C-15 hydroxyl group was epimeric to that found in vertebrates; the major PG constituents were identified as 15*R*-PGA_2 methyl ester acetate and 15*R*-PGA_2 methyl ester ([13] reviewed in [14]). The occurrence of these large quantities of 15*R*-PGs in *P. homomalla* led to intense investigations on its potential as a commercial source of PGs for research and therapeutics [15]. It was discovered that *P. homomalla* collected from other locations such as the Cayman Islands and the Bahamas contain PGs with the 'normal' 15*S*-configuration [16–19]. In rare cases some single specimens were found to contain both 15*R*- and 15*S*-isomers in approximately equal amounts [20].

Due to the inability of *P. homomalla* preparations to biosynthesize PGs *in vitro* [21], the metabolic origin of the unusual 15*R*-PGs remained uncertain until our recent report on the cloning and expression of a COX from the *R*-variety of *P. homomalla* [22]. The discovery of the 15*R*-specific COX in *P. homomalla* confirmed that the PGs of 15*R*-configuration are synthesized directly from arachidonic acid via a 15*R*-PG endoperoxide intermediate and not through isomerization of the 15*S*-hydroxyl. We already knew from cloning and expression experiments in another PG-containing soft coral, the Arctic species *Gersemia fruticosa*, that invertebrates can contain a 15*S*-specific COX enzyme [23,24]. The *P. homomalla* 15*R*-COX shares 80% sequence identity with the *G. fruticosa* enzyme and each is about 50% identical in peptide sequence to mammalian COX-1 and COX-2 [22,23]. The almost certain occurrence of 15*R*-specific and 15*S*-specific COX enzymes in variants of the same species, *P. homomalla*, offered the possibility of comparing two closely related isozymes naturally evolved with opposite C-15 stereocontrol. The aim of the present study therefore was to clone and

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Abbreviations: COX, cyclooxygenase; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid.

Enzyme: prostaglandin-endoperoxide synthase from *Plexaura homomalla* (EC 1.14.99.1; GenBank accession no. AY615733).

Note: The numbering of amino acid residues is according to the sequence of ovine COX-1.

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characterize the COX enzyme from the *S*-variety of *P. homomalla*, compare its primary structure and catalytic properties with its 15*R*-specific counterpart, and locate the residues responsible for control of the C-15 stereochemistry of the products.

Experimental procedures

Materials

Two frozen samples of the *S*-variety of *P. homomalla* collected in the Bahamas (at Sweetings Cay and Cat Island) were obtained as a generous gift from J. Sanchez, SUNY, Buffalo, NY. [1-^{14}C]Arachidonic acid was from Amersham Pharmacia Biotech. Hydroxyeicosatetraenoic acid (HETE) and PG standards were from Cayman Chemical Co. (Ann Arbor, MI, USA). Enzymes, unless otherwise specified, were from Fermentas (Vilnius, Lithuania).

PCR cloning and sequence analysis

RNA was extracted from *P. homomalla* following a previously published protocol [25] except for slight modifications necessary to adjust for the small amount of coral material (< 1 g). For cDNA synthesis, 20 μg total RNA were used with an oligo-dT adaptor primer and murine MLV reverse transcriptase (Promega) as described before [25]. The novel COX cDNA from the *S*-variety of *P. homomalla* was cloned by RT-PCR using primers that exactly matched the first 27 nucleotides and the last 27 nucleotides of the 15*R*-COX ORF from *P. homomalla* (GenBank Accession No. AY004223). The upstream and the downstream primer encoded also a *Bam*HI site and an *Eco*RI site, respectively, for subsequent cloning. For PCR, the Expand High Fidelity kit (Roche) was used following the manufacturer's instructions.

Site-directed mutagenesis

The Ile349Val mutant of the 15*R*-COX and the Val349Ile mutant of the novel 15*S*-COX were constructed using the overlap extension method [26]. The universal mutation primer, 5′-AATTGTGGTGCATTCACAAAAGAGC-3′) was designed to be downstream and the same for both mutants. The specific mutation primers (upstream) were 5′-GTCATTGAAGATTATGTTAACCATCTTGCTA-3′ for the Ile349Val mutant and 5′-GTCATTGAAGAT TATATTAATCATCTTGC-3′ for the Val349Ile mutant. To facilitate the selection of mutated clones, the primers generating the mutations were designed to contain an extra restriction site. A *Hpa*I restriction site for the Ile349Val mutant and a *Vsp*I restriction site for the Val349Ile mutant were designed into the primers without affecting the reading frame or deduced amino acid sequence. (The changed nucleotides are underlined.) The primers used for the full-length clones, 5′-CGATATTGGATCCGTGGAAGA AATGAAGGC-3′ (upstream) and 5′-AAGGATCCTAA AGTTCATCTTTGATGTTTGCCG-3′ (downstream), had extended regions for the *Bam*HI restriction enzyme and a Kozak consensus sequence for eukaryotic protein expression [27]. *Pfu* DNA polymerase (Promega) with proofreading capabilities was used for PCR. The constructs were amplified in *Escherichia coli* DH5α. The correct orientation of the cDNA insert in the vector was confirmed by digestion with *Xba*I and the presence of mutations by digestion with *Hpa*I or *Vsp*I.

Protein expression and preparation of microsomes

Wild-type and mutant *P. homomalla* COX cDNAs were expressed in the Bac-to-Bac baculovirus expression system using the pFASTBAC1 donor vector (Life Technologies, Grand Island, NY). Sf9 cells were cultured at 27 °C in Sf-900II serum-free insect cell medium. Cells were grown in shaking culture in Erlenmeyer flasks with shaking at 120 r.p.m. Cells with a density of $1.5–2 \times 10^6$ cells·mL^{-1} were infected with recombinant baculovirus with a multiplicity of infection (m.o.i) of 0.02. Cells were harvested after 72 h, washed with NaCl/P$_i$, and stored as a pellet at −80 °C. The pellet was resuspended in ice-cold 50 mM Tris/HCl pH8 (1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) and disrupted by sonication (three bursts of 5 s). The low-speed pellet (5 min at 1000 ***g***) was discarded. The supernatant was centrifuged at 100 000 ***g*** for 1 h to yield the microsomal fraction. Expression levels of the wild-type and mutant *P. homomalla* COX proteins were analysed by Western blotting. The microsomal fraction was resolved by SDS/PAGE and transferred electrophoretically to a 0.2 μm nitrocellulose membrane. For detection, a monoclonal antibody raised against rat COX-2 (PharMingen) was used as described previously [22].

Incubations and product analyses

Incubations performed with either Sf9 cell pellet or microsomal fraction gave a similar product composition. When the crude cell pellet was used, the cells were sonicated briefly before incubations. Incubations were performed using the amount of cell pellet or microsomes able to convert 30–50% of arachidonic acid into PGs (about $3–5 \times 10^6$ cells·mL^{-1}). In a standard assay the protein preparation was suspended in 50 mM Tris/HCl pH 8.0 containing 1 μM hematin, 1 mM adrenalin, and in some cases 0.5 mM $SnCl_2$ was added. The reaction was initiated by addition of 50 μM [1-^{14}C]arachidonic acid and incubations were performed for 15 min at room temperature. The reaction mixture was acidified to pH 3.0 and the products were extracted with ethyl acetate. The extract was dried over Na_2SO_4, evaporated to dryness and dissolved in chloroform. TLC was performed using Silica Gel plates (Merck) and a solvent system of benzene/dioxane/acetic acid (10 : 5 : 0.5; v/v/v) or hexane/ethyl ether/acetic acid (3 : 3 : 0.05, v/v/v). Incubation products and unlabelled authentic PG and HETE standards were visualized with an anisaldehyde spray reagent and brief heating at 90 °C [28]. For product quantification, the TLC plate was cut into zones, extracted with methanol, and the radioactivity was measured by liquid scintillation counting as described before [29].

Results

PCR cloning and structural analysis

We obtained *P. homomalla* samples from the Bahamas and confirmed the 15*S*-configuration of the endogenous PGs by

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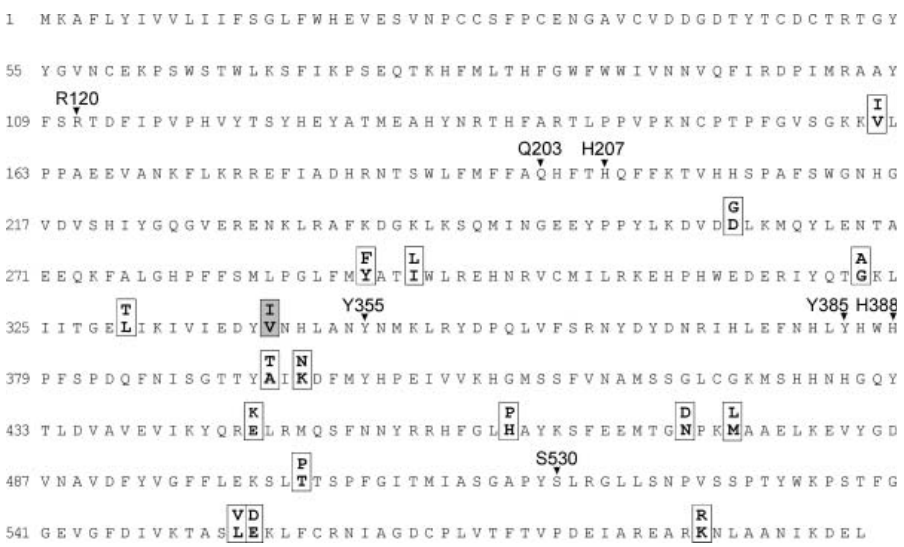

Fig. 1. Deduced amino acid sequence of the novel 15*S*-COX enzyme from *P. homomalla* collected in the Bahamas. Functionally important amino acid residues (R120, Q203, H207, S530 Y355 Y385, H388), conserved between all known COX proteins (given with numeration of ovine COX-1), are marked. In the novel COX sequence, 17 amino acids that are different from the 15*R*-COX of *P. homomalla* from the Florida Keys are boxed, and the respective residues of the 15*R*-COX are given above the sequence. The main determinant of stereospecificity Val/Ile349 is shaded.

HPLC (data not shown). Total RNA was extracted using a protocol optimized for the extraction of difficult samples [25]. Cloning experiments were performed with full-length primers based on the presumed sequence identity with the 15*R*-COX from *P. homomalla*. RT-PCR gave a product of about 1800 bp upon agarose gel electrophoresis, and five of the clones were sequenced entirely. All clones had an ORF of 1776 nucleotides corresponding to 592 amino acids. The deduced amino acid sequence of novel COX is 97% identical with the COX sequence from the same coral species collected in the Florida Keys forming 15*R*-configuration products (Fig. 1). Similar to the 15*R*-specific COX, all amino acid residues shown to be important for substrate binding (Arg120 and Tyr355), hydrogen abstraction (Tyr385), haem orientation and peroxidase activity (His388, Gln203, His207), and aspirin targeting (Ser530) are present in the novel COX cDNA. There are no differences between the two COX proteins in potential N-glycosylation sites nor in the N-terminal cysteines that form the disulphide bonds. The residues that are different between the novel COX and the 15*R*-COX are dispersed along the polypeptide chain with most of them located in the C-terminal half of the protein. Among a total of 17 substitutions only one, Val349 (an Ile in the 15*R*-COX), is located in the cyclooxygenases active site channel [22,30]. The structural model of the novel *P. homomalla* COX, obtained by Swissprot on the basis of three-dimensional data of mammalian COX isozymes (data not shown), reveals that the other 16 residues lie outside of the active site and mostly on the surface of the catalytic globular domain of the COX protein.

Protein expression and product analysis

The cDNA was cloned into the pFASTBAC1 vector for expression in Sf9 insect cells. Products of the *S*-variant COX enzyme were compared with PGs formed by the closely related 15*R*-COX from *P. homomalla*. Incubations of both recombinant COX enzymes with [^{14}C]arachidonic acid were performed using either Sf9 cellular pellet or a microsomal preparation. The products were analysed and quantified by TLC using a solvent system of benzene/dioxane/acetic acid for separation and anisaldehyde reagent for visualization of the spots. The 15*S*- and 15*R*-epimers of PGs were easily distinguishable by their R_f values (Table 1, Fig. 2), PGs of distinct groups also by characteristic colours: PGE, rust brown; PGF, violet; PGD, purple. The two products that migrated closest were PGE_2 and 15*R*-$PGF_{2\alpha}$ (R_f values 0.27 and 0.28, respectively, Table 1). To simplify product analyses, the number of labelled metabolites was decreased by *in situ* reduction of the PG endoperoxide PGG_2 to $PGF_{2\alpha}$ using the mild reducing agent $SnCl_2$. The R_f values for $PGF_{2\alpha}$ (15*S*-isomer) and its 15*R*-epimer are 0.17 and 0.28, respectively, allowing for precise determination of the configuration of carbon-15. The content of monohydroxy acids (HETE) was determined using a solvent system of hexane/ethyl ether/acetic acid. The HETEs accounted for

Table 1. R_f values of PGs and HETEs formed from arachidonic acid in incubations with 15*S*- and 15*R*-COX enzymes. TLC analyses were performed on silica plates using a solvent system of benzene/dioxane/acetic acid (10 : 5 : 0.5, v/v/v). Products were visualized with anisaldehyde spray reagent followed with brief heating at 90 °C.

	R_f values	
Compound	15*S*-epimer	15*R*-epimer
$PGF_{2\alpha}$	0.17	0.28
PGE_2	0.27	0.38
PGD_2	0.46	0.54
Monohydroxy acids	0.75	
Arachidonic acid	0.84	

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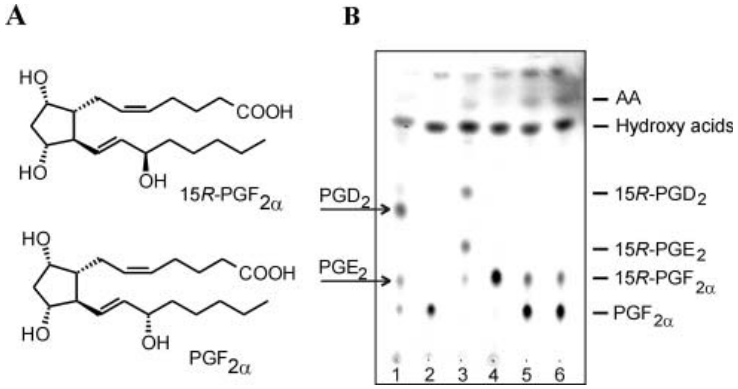

Fig. 2. TLC analysis of products formed from [^{14}C]arachidonic acid by wild-type and mutant *P. homomalla* COX proteins expressed in Sf9 cells. (A) Structures of $PGF_{2\alpha}$ and 15*R*-$PGF_{2\alpha}$. (B) TLC separation of incubation products. Incubations of [1-^{14}C]arachidonic acid with recombinant coral COX proteins were carried out as described in Experimental procedures. TLC was performed using silica gel plates and a solvent system of benzene/dioxane/acetic acid (10 : 5 : 0.5, v/v/v). The products were visualized with an anisaldehyde spray reagent. Lanes: 1 and 2, wild-type 15*S*-COX; lanes 3 and 4, wild-type 15*R*-COX; lane 5, Ile349Val mutant of 15*R*-COX; lane 6, Val349Ile mutant of 15*S*-COX. In lanes 2, 4, 5 and 6, the incubations were performed in the presence of 0.5 mM $SnCl_2$.

Table 2. Stereochemical composition of labelled $PGF_{2\alpha}$ formed from [^{14}C]arachidonic acid by wild-type and mutant *P. homomalla* COX enzymes. The percentage given is a mean value of at least three different expressions.

	Content of $PGF_{2\alpha}$ epimers (%)	
Recombinant COX	15*S*-epimer	15*R*-epimer
Wild-type 15*S*-COX	98	2
Val349Ile 15*S*-COX	65	35
Wild-type 15*R*-COX	2	98
Ile349Val 15*R*-COX	70	30

< 10% of the total labelled products, with 11-HETE as the main component (data not shown).

TLC analysis of the reduced incubation products showed that the novel 15*S*-COX enzyme formed 98% 15*S*-$PGF_{2\alpha}$, while the previously cloned (recombinant) 15*R*-COX from *P. homomalla* formed 98% 15*R*-$PGF_{2\alpha}$ (Table 2). Thus, the products formed by COX enzymes from the two variants of *P. homomalla* match their respective endogenous content of PGs.

Mutational analysis

A prime candidate for the control of oxygenation stereospecificity at carbon-15 is the active site residue at position 349, Val in the novel *P. homomalla* 15*S*-COX and in all known 15*S*-specific COX proteins, and an Ile in the 15*R*-COX [22,31] To determine the role of residue 349 in the specificities of the two *P. homomalla* variants, a Val349Ile mutant of the 15*S*-COX and an Ile349Val mutant of the 15*R*-COX were prepared and expressed in the baculovirus system. The mutants were incubated with [1-^{14}C]arachidonic acid in the presence of $SnCl_2$ and the products were analysed by TLC (Figs 2 and 3, and Table 2). Equivalent amounts of wild-type and mutant proteins as quantified by Western analysis gave similar conversion of the radiolabelled substrate. The Val349Ile mutant of the 15*S*-COX formed 65% $PGF_{2\alpha}$ and 35% of the 15*R*-epimer of $PGF_{2\alpha}$. In the case of the 15*R*-COX, the Ile349Val mutation caused a more pronounced effect on the stereochemistry of oxygenation, inverting the configuration from 98% 15*R*-$PGF_{2\alpha}$ to 70% $PGF_{2\alpha}$.

Discussion

The studies reported here present structural differences between COX enzymes from the two variants of *P. homomalla* and establish that they form PGs with opposite stereochemistry at C-15. The primary structures of the 15*R*- and 15*S*-COX variants of *P. homomalla* share 97% sequence identity and differ in only 17 amino acids. Based on the strong homology and the known three-dimensional structures of mammalian COX proteins, only one of these 17 amino acids impinges directly into the oxygenase active site. This highly conserved residue, Val349, has been characterized as one of the critical residues along with Trp387 and Leu534 that contribute to the positioning of arachidonic acid in a conformation such that when hydrogen abstraction occurs the substrate is appropriately arranged to yield PG endoperoxide [32]. The authors explained the role of Val349 through stabilization of the carboxyl half of arachidonic acid to promote proper positioning of C-9 with respect to C-11, necessary for cyclopentane ring formation. Ovine COX-1 mutants in which Val349 was replaced with residues such as alanine, serine or threonine, produced an abundance of 11*R*-HETE vs. PGs. On the other hand, replacing of Val349 with the more bulky leucine led to formation of a relatively large amount of 15-HETE [32].

The residue 349 was implicated earlier in C-15 stereocontrol by its occurrence as Ile349 in the *P. homomalla* *R*-COX in place of the conserved Val349 [22]. Subsequently, the Val349Ile mutation was tested for its influence on PG stereochemistry in human COX-1 and COX-2 and found

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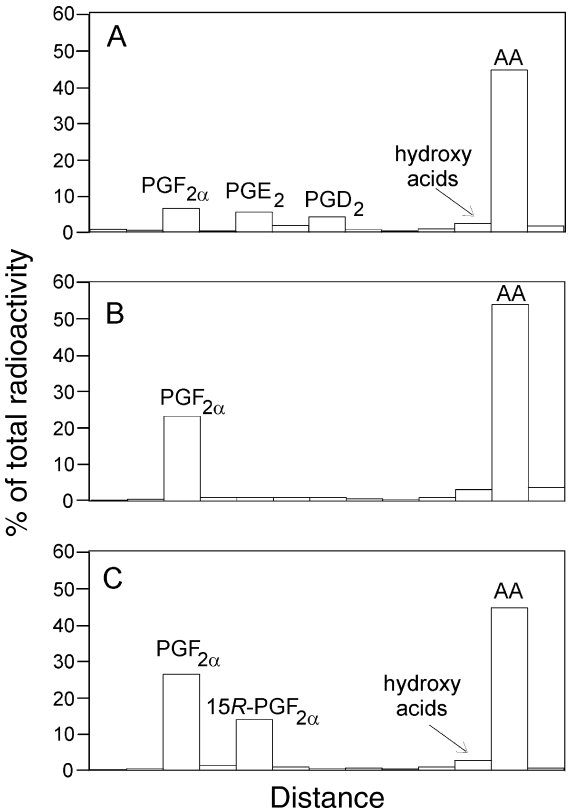

Fig. 3. TLC radiochromatograms of the products formed from [^{14}C]arachidonic acid with wild-type and mutant *P. homomalla* 15*S*-COX expressed in Sf9 cells. Incubations of [1-^{14}C]arachidonic acid with recombinant coral COX proteins were carried out as described in Experimental procedures. (A) Wild-type 15*S*-COX; (B), wild-type 15*S*-COX with the incubation performed in the presence of 0.5 mM $SnCl_2$; (C) Val349Ile mutant of 15*S*-COX incubated in the presence of 0.5 mM $SnCl_2$. The incubation products were separated with a solvent system of benzene/dioxane/acetic acid, 10 : 5 : 0.5, v/v/v. TLC plates were cut into 13 zones, extracted with methanol, and the radioactivity was determined by liquid scintillation counting.

to partially switch the C-15 configuration. Site-directed mutagenesis of Val349 in human COX-1 and COX-2 to Ile yielded enzymes that formed 41% and 60–65% 15*R*-PGs, respectively [31]. We found here that the COX from the *S*-variety of *P. homomalla* contains a Val349, in line with all the other *S*-specific isozymes. While the wild-type *R*- and *S*-variant COX enzymes formed almost pure 15*R*- and 15*S*-PGs, respectively, changing Val349 to Ile and vice versa had a greater effect on the *R*-COX. Whereas the mutant 15*R*-COX formed 70% 15*S*-PGs, the mutant 15*S*-COX formed only 35% 15*R*-PGs. The latter result is in good accord with the results of the Val349Ile mutation of human COX-1 [31]. This partial inversion of the product stereochemistry in the single-residue mutants implies the contribution of other, as yet unidentified residues in oxygenation stereocontrol. Due to their extremely high structural identity, the pair of *P. homomalla* COX isoforms serves as an ideal model for further elucidation of residues involved in oxygenation stereocontrol in COX catalysis.

The occurrence of colonies of *P. homomalla* containing either 15*R*- or 15*S*-PGs raises several issues. The biological function of the high PG content (2–3% of the coral dry weight [19]) is unlikely to be a signalling role in the usual sense. Such high concentrations of PG methyl esters cannot be in true solution and probably exist in a separate lipid phase. Furthermore, if the PGs were to function as signalling molecules, one might expect that receptor targets should also have evolved to preferentially respond to either 15*R*- or 15*S*-PGs. It seems more likely that the corals with extremely high PG content use these lipids in biodefence. It has been proposed that *P. homomalla* use PGs as protective substances against predation from feeders [33]. Several studies support this hypothesis. Many fish that digest food pellets that contain lipid extracts of this coral would become ill and vomit. After several attempts fish rejected subsequent offers of treated pellets. The antifeeding effect of totally esterified PGs as they naturally occur in the coral is slower as they become active only after partial hydrolysis (see [34] for a review).

The finding of COX genes in the 'PG-containing corals' *P. homomalla* and *G. fruticosa* is a sure sign that an equivalent gene is present in other ascidians. Here it seems quite possible that low levels of PGs are used in a more traditional signalling role. Another open issue is the explanation for those rare *P. homomalla* colonies containing similar quantities of 15*R*- and 15*S*-PG. The physical organization of all corals, including *P. homomalla*, comprises hundreds or thousands of individuals in a colony. If a mixture of PGs is found, do some animals express the *R*-specific COX and others the *S*-specific variant? An intriguing alternative is that the colony contains yet another variant COX protein that functions with less stringent stereocontrol for the oxygenation at C-15, for example, an otherwise *S*-specific COX mutated to an Ile at amino acid position 349.

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