A Critical Role of Non-active Site Residues on Cyclooxygenase Helices 5 and 6 in the Control of Prostaglandin Stereochemistry at Carbon 15*

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The correct stereochemistry of prostaglandins is a prerequisite of their biological activity and thus is under a strict enzymatic control. Recently, we cloned and characterized two cyclooxygenase (COX) isoforms in the coral Plexaura homomalla that share 97% amino acid sequence identity, yet form prostaglandins with opposite stereochemistry at carbon 15. The difference in oxygenation specificity is only partially accounted for by the single amino acid substitution in the active site (Ile or Val at position 349). For further elucidation of residues involved in the C-15 stereocontrol, a series of sequence swapping and site-directed mutagenesis experiments between 15R- and 15S-COX were performed. Our results show that the change in stereochemistry at carbon 15 of prostaglandins relates mainly to five amino acid substitutions on helices 5 and 6 of the coral COX. In COX proteins, these helices form a helix-turn-helix motif that traverses through the entire protein, contributing to the second shell of residues around the oxygenase active site; it constitutes the most highly conserved region where even slight changes result in loss of catalytic activity. The finding that this region is among the least conserved between the P. homomalla 15S- and 15R-specific COX further supports its significance in maintaining the desired prostaglandin stereochemistry at C-15. The results are particularly remarkable because, based on its strong conservation, the conserved middle of helix 5 is considered as central to the core structure of peroxidases, of which COX proteins are derivatives. Now we show that the same parts of the protein are involved in the control of oxygenation with 15R or 15S stereospecificity in the dioxygenase active site.

In vertebrates, the conversion of polyunsaturated fatty acids to prostaglandins (PG)2 is catalyzed by PG endoperoxide synthase3 or prostaglandin H (PGH) synthase, also known as cyclooxygenase (COX) (1). Two structurally and mechanistically similar isoforms of COX have been identified in vertebrates, the constitutively expressed COX-1 and the inducible COX-2 (2–5). The two enzymes share a high degree of amino acid sequence identity (60–65%) and structural homology (root mean square deviation <1.0 Å) (6–8). Both isoforms catalyze a similar reaction that involves the bisoxygenation of arachidonic acid to form prostaglandin G2 followed by a reduction step to form prostaglandin H2 (9). Subsequent cell-type specific metabolism of PGH2 results in the production of the primary prostaglandins (E2, D2, and F2α), prostacyclin, and thromboxane A2. These bioactive molecules all retain the 15S configuration of PGH2 and are utilized in signaling by vertebrates from fish to mammals. The correct stereochemistry of the product is crucial for the biological activity and thus is under strict enzymatic control (10).

Although almost all natural prostaglandins occur with the 15S stereoconfiguration, there is one well documented exception. The endogenous prostaglandins extracted from the Caribbean coral Plexaura homomalla exhibit either the usual 15S or the unique 15R configuration, depending on the geographical location (11–13). The COX enzyme cloned from P. homomalla samples collected in the Bahamas synthesizes prostaglandins with the regular 15S configuration, whereas the enzyme from the same species collected in the Florida Keys forms prostaglandins with the unique 15R configuration (14, 15). These two enzymes share 97% amino acid identity, yet they perform the oxygenation reaction of arachidonic acid with opposite stereoselectivity at carbon 15. The finding that highly similar enzymes yield products with opposite stereochemistry presents an excellent opportunity to determine the role of individual amino acid residues in maintaining the correct stereochemistry of the product. There is only one active site amino acid that differs between the 15R- and 15S-COX, Ile or Val at position 349, respectively, in the two isoforms, yet it is only partially responsible for the C-15 stereocontrol. The V349I mutant of the S-variety P. homomalla COX switched only 35% toward 15R-prostaglandin synthesis, retaining 65% of products in the 15S configuration. The converse mutant (I349V) of R-variety coral COX started to produce prostaglandins with 70% of S-configuration (14, 15). Similar results have been reported when Val-349 of human COX-1 and COX-2 was replaced with isoleucine; the mutant enzymes formed 41 and 65% 15R-prostaglandins, respectively (16).

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‡The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; TLC, thin layer chromatography; PCR, polymerase chain reaction; RT, reverse transcriptase; RP-HPLC, reverse phase high pressure liquid chromatography.

3The enzyme prostaglandin-endoperoxide synthase is from Plexaura homomalla (EC 1.14.99.1).
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The aim of the present study was to uncover other residues involved in stereocntrol of the second oxygenation. Using sequence swapping as well as site-directed mutagenesis to replace segments or individual amino acid residues of 15R-COX with the corresponding sequences or particular residues of 15S-COX, several chimeras and mutant enzymes were produced and analyzed for product stereochemistry.

EXPERIMENTAL PROCEDURES

Materials—The origin and preparation of recombinant 15S- and 15R-specific COX enzymes from P. homomalla has been described previously (14, 15). [1-14C]Arachidonic acid was purchased from Amersham Biosciences. Prostaglandin standards and arachidonic acid were from Cayman Chemical Co. (Ann Arbor, MI). Restriction enzymes were obtained from Fermentas (Vilnius, Lithuania), Taq polymerase from Naxo Ltd. (Tartu, Estonia), and Pfu polymerase from Promega. The other reagents were from Sigma or Aldrich. Primers were from DNA Technology A/S (Aarhus, Denmark). Plasmid DNA was purified using a Qiaprep spin miniprep kit (Qiagen, Valencia, CA) for pFASTBAC1 and pGEM-T-Easy, or JETstar Mini (Genomed GmbH) for bacmids.

Mutagenesis by Swapping Parts of DNA Sequences (Chimeragenesis)—15S- and 15R-specific P. homomalla COX genes were cloned into the BamHI site of the pFASTBAC1 vector (Invitrogen). The chimeric constructs were obtained using common restriction sites. Vectors containing the coding region of the 15S- or 15R-specific COX or one of the mutant constructs were digested with appropriate restriction enzymes (BamHI, NcoI, XbaI, or BanI) and the corresponding fragments swapped between the counterparts. The mutations were performed within the pFASTBAC1 vector. In the case of BanI that cuts pFASTBAC1 vector thrice, subcloning into the pGEM-T-Easy vector (Promega) was used. The vector parts were dephosphorylated with calf intestine alkaline phosphatase (Fermentas), ligated with T4 DNA ligase (Promega), and transformed by heat shock into Escherichia coli DH5α cells. The success of swapping was verified by restriction analysis with enzymes that cut the 15S- and 15R-specific COX genes with different product patterns. BclI cuts the 15R-specific gene once and the 15S-specific gene twice and does not cut the pFASTBAC1 vector. The extra site is located in the middle of the 15S-specific COX gene (after nucleotide 989). The 15R-COX gene has an extra HindIII restriction site close to the 3’ end (after nucleotide 1659).

Site-directed Mutagenesis—Site-directed mutations were performed by PCR using the overlap extension method (17). Generation of the mutants M1 and M2 is described before (15). Other site-directed mutants (M9 — M20) were obtained in a similar way. The universal mutation primer was the same used for M1 and M2, and the specific mutation primers were designed to contain extra restriction sites for selection of mutated clones (Hpal, Stul, HindIII, and VspI). The PCR-generated fragments were inserted into the pFASTBAC1 vector at the BamHI restriction site. The mutations were sequenced.

Protein Expression and Enzyme Assay—DH5α cells were used for the propagation of plasmid DNA. The positive clones were transposed to bacmids according to the manufacturer’s protocol and expressed in Sf9 cells as described previously (14). The cells were harvested 72 h post infection, washed with phosphate-buffered saline, and stored as a pellet at −80 °C. The expression level of protein was assessed by Western blotting using a monoclonal antibody raised against rat COX-2 (Pharmlingen) as described previously (Fig. 1) (15). The relative activities of wild-type and mutated enzymes were compared by incubations with radiolabeled substrate (18). Equal amounts of cells expressing either wild-type or mutant COX were assessed for ability to convert arachidonic acid to PGF2α, under reducing conditions as described below. The incubations were conducted in conditions where the conversion of arachidonic acid did not exceed 30%.

Frozen cell pellets were suspended in 50 mM Tris/HCl (pH 8.0) containing 1 μM hematin and 1 mM adrenaline and preincubated for 2 min at room temperature. The reaction was initiated by addition of 50 μM [1-14C]arachidonic acid, and the incubations were performed at room temperature for 10 min. Product stereochemistry was measured by in situ reduction of the primary prostaglandin endoperoxide product of the COX enzymes (PGG2) to a PGF2α, with carbon 15 being in either S- or R-configuration. Reduction was effected by a 1 mM aqueous suspension of reducing agent SnCl2 added to the incubation mixture directly before the substrate. Reaction mixtures were acidified and the products extracted with ethyl acetate. Radioactive products were separated by thin layer chromatography (TLC) using silica gel plates (Merck) and a solvent system of benzene/dioxane/acetic acid (5/2.5/0.25, v/v/v). The products were visualized with anisaldehyde and quantified by liquid scintillation counting.

Cyclooxygenase Assay—Oxygen consumption by COX enzymes was determined using a fiber optic oxygen monitor, model 110 from Instech Laboratories Inc. The total reaction volume was 230 μl (50 mM Tris/HCl, pH 8.0, 1 mM adrenaline). The reaction was initiated by addition of arachidonic acid solu-
tion in ethanol so that the added volume did not exceed 1%. The assays were conducted in a thermostated reaction chamber at 25 °C. When cell homogenates were assayed then maximum amount of cells analyzed was 1.5 million.

Microsomal fractions were used to determine the $K_m$ values. To obtain microsomal membranes, the cell pellets were thawed, resuspended in 50 mM Tris/HCl, pH 8.0, 1 mM adren- 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and sonicated 3 × 5 s with 30 s intervals for cooling. The sonicates were centrifuged at 1000 g for 5 min. The pellets were washed and recentrifuged. The supernatants were combined and centrifuged at 200,000 g for 30 min to get microsomal membranes. The amount of microsomes used for analysis was selected so that the $V_{max}$ was 30 – 80 μM O₂/min; the concentration of arachidonic acid was varied between 2 and 200 μM. $K_m$ values were calculated from the Michaelis-Menten equation using the Sigma Plot 8 program.

**RESULTS**

The $K_m$ values were determined for arachidonic acid with microsomal fractions of both 15S- and 15R-specific wild-type enzymes and one mutant enzyme exhibiting dual enantioselec- tivity (M2). The $K_m$ values were 5.8 ± 1.7 μM for native 15S-COX, 8.7 ± 2.4 μM for native 15R-COX, and 8.3 ± 1.8 μM for the M2 (V349I) mutant of 15S-COX. Thus the two coral COX enzymes have similar kinetic properties (with arachidonic acid as substrate) despite the fact that their products are diastereoi-

**Stereospecificity of Oxygenation Reaction**—Stereoisomers of PGF₂α are well separable with TLC system described above. The Rf values of PGF₂α are 0.17 for the 15S-epimer and 0.28 for the 15R-epimer (14). Similar results were obtained when the radioactive products were analyzed by reverse phase high performance liquid chromatography using a Stellar Phases C₁₈ column (25 × 0.46 cm) and Radiomatic Flo-One detector. When methanol or acetonitrile alone was used as organic modifiers the carbon 15 stereoisomers of PGF₂α were unseparable, whereas an equimolecular mixture of the two gave good reso-

**Chimeric 15R/15S-COX Enzymes**—The high sequence identity between 15S- and 15R-specific COX facilitated the use of common restriction sites to swap parts of the cDNAs. (Fig. 2) Exchanging the cDNA flanked by two NcoI restriction sites swapped the amino acids from Tyr-138 to His-440, a region of the proteins accounting for nine of the seventeen amino acid differences. The resulting chimeric COX pro-

**FIGURE 2. A partial restriction map and sequence swapping strategy of 15S- and 15R-cyclooxygenases from P. homomalla.** P. homomalla COX genes were digested with restriction enzymes shown on the figure, and the sequences were swapped between the counterparts. The resulting mutants M3–M8 are given as schematic drawings where lighter areas represent parts of sequences derived from the 15R-COX, and the darker areas are those of the 15S-COX.

**FIGURE 3. Separation of stereoisomers of PGF₂α by RP-HPLC.** The mutant enzymes were expressed in insect cells and analyzed for product stereochro-

15R PGF₂α were analyzed either by TLC (as described under “Experimental Procedures”) or by RP-HPLC (Fig. 3). Analysis of the 15S-COX chimera with nine changed residues (mutant M3) indicated that it formed 85% of 15R-prostaglandins, and the analogous 15R-specific COX (mutant M4) formed almost exclusively 15S prostaglandins (>95%) (Table 1).

The nine changes in mutants M3 and M4 were narrowed down to seven by making use of the common XbaI restric-

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| Table 1 |
|---------|
| Mutant | Product Epimer Ratio |
| M3 | 85% 15R PGF₂α |
| M4 | 95% 15S PGF₂α |

Baculovirus expression of these new constructs followed by incubation with [¹⁸C]ar-
achidonic acid and product analysis indicated that the second series mutants M5 and M6 produced approximately the same composition of products as M3 and M4, meaning that amino acid residues 170 and 271 had no significant influence on oxygenation stereospecificity. In a similar way the influence of amino acid residues 403 and 405 was excluded using the common BanI restriction site and the common BamHI cloning site at the 3′/H11032 end of the vector (Table 1). The resulting chimeric enzymes were interchanged in their C-terminal regions beginning from amino acid Trp-387 (mutants M7 and M8). The chimera with the N-terminal part of 15S-COX and the C-terminal part of 15R-COX (M8) gave a product profile similar to the regular 15S-specific COX. The opposite case, the chimera with R-specific N- and S-specific C terminus (M7) formed prostaglandins with 85% of R- and 15% of S-configuration. Comparison of the product composition with those from the aforementioned mutants (M3 and M4) precluded the influence of amino acid residues 403 and 405.

Altogether the results of the sequence swapping experiments indicate that the S-configuration of carbon 15 of the prostaglandins is determined by the section of protein including five out of the seventeen amino acid differences between 15S-COX and 15R-COX. Homology modeling of the enzyme structures (Fig. 4) shows that this protein region consists of two consecutive helices, helices 5 and 6. (The nomenclature for the helices is underlined.)

**TABLE 1**

| 15R-COX | 15S-COX | template | mutation restriction | %S | %R | activity |
|--------|--------|----------|---------------------|---|---|---------|
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M1 | Ile349Val | >5 | <5 | +++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M2 | Val349Ile | 65 | 35 | +++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M3 | M3+Swt | 15 | 85 | ++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M4 | M3+Swt | 15 | 85 | ++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M5 | M3+Swt | 20 | 80 | ++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M6 | M4+Swt | 15 | 85 | ++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M7 | M4+Swt | 15 | 85 | ++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M8 | M4+Swt | 15 | 85 | ++ |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M9 | M7 | XbaI | >5 | <5 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M10 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M11 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M12 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M13 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M14 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M15 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M16 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M17 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M18 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M19 | M7 | XbaI | 15 | 85 |
| IGFLATINKPDLPVRD | VDYIGLVAKEHNMTLEK | M20 | M7 | XbaI | 15 | 85 |

**FIGURE 4.** Model of amino acid differences between 15S- and 15R-specific COX. The modeling was done using Swiss model program and RasMol for visualization. Locations of amino acid differences are shown on ovine COX-1 (1DIY), along with catalytically competent arachidonic acid (spacefill mode) bound in the cyclooxygenase active site and heme (wireframe mode) in the peroxidase site. The membrane-binding domain (MBD) and epidermal growth factor-like (EGF) domain are darker gray. Amino acid residues Arg-120 and Tyr-385 important for substrate binding and catalysis are in wireframe mode. Two successive helices (helices 5 and 6), the main determinants of stereospecificity of carbon 15, are in black. The amino acid differences on helices 5 and 6 are underlined.
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FIGURE 5. A multiple sequence alignment of COX helices 5 and 6. Residue numbering follows the convention for the ovine COX-1 structure. An asterisk under a given amino acid marks the absolute conservation in that position. Amino acid differences between 15S- and 15R-cyclooxygenases are shadowed.

based on the spatial equivalence of the secondary structure between myeloperoxidases (6)). Exchange of the two helices in the 15R-COX evokes a complete switch in product stereochemistry to 15S, whereas the analogous replacement in the 15S-specific COX gives a chimera, which switches stereochemistry by 85%, whereas continuing to form a small percentage of 15S-prostaglandins (~15%).

Mutations within Helices 5 and 6—To figure out which amino acids among those five (besides Val-349/Ile-349) play a role in the stereocontrol, a series of site-directed mutations were prepared with amino acids exchanged between counterparts. The first mutations were done with two amino acids residing on the same helix as Val-349/Ile-349 (helix 6), alanine (15R) versus glycine (15S) at position 332 and threonine (15R) versus leucine (15S) at 340. The multiple sequence alignment of COX sequences from coral to mammals (Fig. 5, with all but the 15R P. homomalla variant being 15S-COX) shows that Thr-340 is a strongly conserved residue. Remarkably, it is the S-variety of P. homomalla that differs in having a Leu-340 (and the 15S-cyclooxygenase (M19), a chimeric enzyme in which helix 6 was replaced with the 15S sequence, was inactive. This is probably because of the aforementioned unfavorable threonine-leucine interchange at position 340. The converse mutant (M20, a 15S-variety COX with helix 6 of the 15R-COX) was fully active with the stereochemistry of the products nearly the same as that of the single mutant I349V (M1). Single mutants of position 332, G332A of S-specific and A332G of R-specific cyclooxygenase (mutants M17 and M18) were also made. Surprisingly, both mutants remained fully active but had no change in oxygenation stereospecificity.

An attempt was made to restore the activity of the M13 and M15 mutants of 15R-COX by changing the whole set of three residues on the helix 6, but the triple mutant of the R-specific cyclooxygenase (M19), a chimeric enzyme in which helix 6 was replaced with the 15S sequence, was inactive. This is probably because of the aforementioned unfavorable threonine-leucine interchange at position 340. The converse mutant (M20, a 15S-variety COX with helix 6 of the 15R-COX) was fully active with the stereochemistry of the products nearly the same as that of the single mutant M2. The results indicated that the additional stereospecificity determinants have to reside on helix 5.

On helix 5 the differences between the two P. homomalla cyclooxygenases are in two amino acids Phe-301/Tyr-301 and Leu-304/Ile-304, 15R/15S, respectively. These residues were mutated pairwise, which means that besides V349I the whole helix 5 was changed (mutants M11 and M12), and also position 301 alone was exchanged (M9 and M10). In all cases except one (M9) the mutant enzymes were inactive. The composition of products of the double mutant M9 (F301Y, I349V of 15R-COX) did not significantly differ from that of the single I349V mutant of the 15R-COX (M1).

The conclusion is that the major amino acid determinants of oxygenation stereospecificity stand on the pair of helices 5 and 6 and that helix 5, which lies quite far from the active site, contributes substantially to the stereospecificity of the oxygenation reaction at C-15.
DISCUSSION

The very similar 15R- and 15S-COX genes in the two varieties of *P. homomalla* are ideally suited for chimeragenesis and sequence-swapping experiments. Each of these enzymes contains all the conserved amino acids that play a vital role in cyclooxygenase catalysis across all animals from corals to humans. This includes the well studied catalytic Tyr-385, the Ser-530 that is acetylated by aspirin, the Arg-120 and Tyr-355 that help form a gateway into the substrate binding channel, and residues involved in coordination of the heme iron or in peroxidase catalysis. The question we were able to address here, by making use of the unique 15R-COX, is what structural features controlled the usual 15S stereochemistry of the prostaglandins? Of the total of seventeen amino acids that differ between the 15R- and 15S-COX of *P. homomalla*, previously we were able to implicate Val-349/Ile-349 (15), the only one of the seventeen that lines the oxygenase active site (21, 22). The other sixteen residues are dispersed all over the protein, far from the active site, mostly on the surface of the catalytic domain (Fig. 4). Therefore, to help define parts of the primary structure that may influence carbon 15 chirality, several chimeras between *S*- and *R*-variety *P. homomalla* COX sequences were prepared. Several highly active mutants that formed mixtures of prostaglandins with different ratios of 15S and 15R isomers were obtained (Table 1). The ratio of the isomers did not depend on substrate concentration. Formation of the two epimers was also blocked by inhibitors at similar rates (data not shown).

Analysis of the chimeras and further point mutations indicated that crucial 15S stereospecificity determinants lay on the helices 5 and 6. Those two long helices form a helix-turn-helix motif (23) and constitute a frame to the whole catalytic domain. Alignment of the primary sequences of cyclooxygenases from different organisms demonstrates that those helices are the most highly conserved regions in COX proteins.

The sequence identity of the whole enzyme between COX-1 and COX-2 is 60–65% within the same species whereas the identity among orthologs from different vertebrate species is among the least conserved parts of the entire protein between the 15S- and 15R-cyclooxygenases. Five of the seventeen differences between the isoenzymes are situated on those two helices, which means that compared with the 97% overall identity, the conservation within helices 5 and 6 is only 90%. This is probably not accidental, but reflects the importance of helices 5 and 6 in maintaining the desired carbon 15 stereochemistry in the two variants of the *P. homomalla* COX proteins.

In summary, in this paper we have succeeded in specifying the individual residues involved in the stereocatalysis of the second oxygenation reaction of COX proteins. We conclude that the structural helix-turn-helix motif (helices 5 and 6) plays a crucial role in determining the configuration at carbon 15 of resulting prostaglandin products. Because of the frequency with which inactivating mutations are encountered within those helices we could not specify the exact role of each single amino acid residue. It appears that these structures act in a concerted manner, and that helix 5, despite its distance from the active site, has an important role in the control of prostaglandin stereochemistry. It is particularly remarkable because the absolutely conserved region in the middle of the helix 5 is more associated with it constituting a crucial structural feature of all peroxidases. Helices 5 and 6 were thought to be just central building blocks for a peroxidase body. Now we show that the same parts of the protein are also involved in the control of oxygenation with 15R and 15S stereospecificity in the dioxygenase active site.

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