Stereospecificity of Hydrogen Abstraction in the Conversion of Arachidonic Acid to 15R-HETE by Aspirin-treated Cyclooxygenase-2

IMPLICATIONS FOR THE ALIGNMENT OF SUBSTRATE IN THE ACTIVE SITE*

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The initial and rate-limiting step in prostaglandin biosynthesis is stereoselective removal of the pro-S hydrogen from the 13-carbon of arachidonic acid. This is followed by oxygenation at C-11, formation of the five-membered ring, and a second oxygenation at C-15 to yield the endoperoxide product, prostaglandin G2. Aspirin treatment of cyclooxygenase-2 is known to acetylate an active site serine, block prostaglandin biosynthesis, and give 15R-hydroxyeicosatetraenoic acid (15R-HETE) as the only product. 15R-HETE and prostaglandins have opposite stereoisomers of the 15-hydroxyl. To understand the changes that lead to 15R-HETE synthesis in aspirin-treated COX-2, we employed pro-R- and pro-S-labeled [13-3H]arachidonic acids to investigate the selectivity of the initial hydrogen abstraction. Remarkably, aspirin-treated COX-2 formed 15R-HETE with removal of the pro-S hydrogen at C-13 (3–9% retention of pro-S tritium label), the same stereoselectivity as in the formation of prostaglandins by native cyclooxygenase. To account for this result and the change in oxygenase activity, we suggest that the bulky serine acetyl group forces a realignment of the omega end of the arachidonic acid carbon chain. This can rationalize abstraction of the C-13 pro-S hydrogen, the blocking of prostaglandin synthesis, and the formation of 15R-HETE as the sole enzymatic product.

In 1967, Hamberg and Samuelsson (1) established that cyclooxygenase initiates prostaglandin biosynthesis by abstraction of the pro-S hydrogen from the 13-carbon of the polynsaturated fatty acid substrate. This is followed by insertion of two molecules of oxygen at positions 11 and 15 on the carbon chain to form the endoperoxide product PGG2. In terms of stereochemistry, both of these oxygenations are on the opposite face of the substrate with respect to the hydrogen removed at C-13 (1). This so-called antarafacial relationship between hydrogen abstraction and oxygenation has proved to be the rule for all stereospecific reactions of cyclooxygenases (COX) and lipoxygenases investigated so far (e.g. Refs. 1–3).

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The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HHT, 12-hydroxyheptadecatrienoic acid; RP-HPLC, reversed-phase high pressure liquid chromatography; SP-HPLC, straight-phase high pressure liquid chromatography.

Non-steroidal anti-inflammatory drugs are well known as inhibitors of the two mammalian cyclooxygenase enzymes, COX-1 and COX-2 (4, 5). In contrast to all other NSAIDs, aspirin is known to modify covalently COX-1 and COX-2 by acetylation of a critical serine residue that lines the cyclooxygenase active site (6–9). Acetylation of Ser-530 (using the numerical designation of this serine in ovine COX-1) results in complete inhibition of prostaglandin synthesis by both COX-1 and COX-2. With COX-2 only, the effect of aspirin treatment is a shift in reaction specificity; prostaglandin synthesis is blocked and 15R-hydroxyeicosatetraenoic acid (15R-HETE) is formed as the only enzymatic product (10–12). This reaction is particularly unusual in that 15R-HETE has the opposite stereoisomer at carbon-15 to prostaglandin G2 and some other prostaglandins. A question arises whether the formation of the 15R configuration product is associated with removal of the opposite hydrogen at C-13 as would be predicted from the typical antarafacial relationship (Fig. 1). Either way, a knowledge of the stereochemistry of the hydrogen removal will lend some insights into the mode of substrate binding in the cyclooxygenase active site.

With these considerations in mind we synthesized [13-pro-R-3H]- and [13-pro-S-3H]arachidonic acids by a combined chemical and biosynthetic approach, and we employed these substrates to address the question of hydrogen removal in 15R-HETE synthesis by aspirin-treated COX-2. To explain our experimental results, we propose a model of arachidonic acid binding that involves a changed conformation induced by the acetylation of Ser-530.

EXPERIMENTAL PROCEDURES

Materials—Vaccenic acid methyl ester was purchased from NuChek Prep Inc. (Elysian, MN). [1-14C]Stearic acid (56 mCi/mmol) and [1,14C]arachidonic acid were from NEN Life Science Products. Aspirin (acetylsalicylic acid) was from Aldrich, as were the other reagents used for chemical synthesis. All solvents used for chromatography were of HPLC grade.

Synthesis of [13-pro-R-3H]- and [13-pro-S-3H]Arachidonic Acids—A detailed description of the synthesis of the [13-pro-R-3H]- and [13-pro-S-3H]arachidonic acids will be reported elsewhere. Briefly, the synthesis was accomplished using the following scheme: (i) singlet oxygenation of methyl vaccenate (11Z-octadecenoic methyl ester), (ii) reduction of the 11-hydroperoxyoctadec-12E-enoate and 12-hydroperxyoctadec-10E-enoate to the hydroxy derivatives, (iii) alkaline hydrolysis of the methyl ester and isolation of the later eluting 11-hydroxy acid by SP-HPLC, (iv) oxidation to the keto derivative, (v) removal of the double bond by catalytic hydrogenation, (vi) reduction with NaBH4, (vii) tosylation, (viii) resolution of the enantiomers by chiral phase HPLC (Chiralpak AD), (ix) displacement of the tosylate with LiAlH4, (x) reoxidation at C-1 with chromic acid, (xi) co-culture of the resulting 11-hydroperoxyoctadec-10E-enoate methyl ester, (xii) Preparation of Acetylated COX-2—Baculovirus-expressed wild-type mouse COX-2 was a gift from Drs. Scott Rowlinson and Lawrence J. Marnett (Vanderbilt University) (13). The enzyme (final concentration
Mechanism of 15R-HETE Synthesis by Acetylated COX-2

15R-3H]arachidonic acids were admixed with [3-14C]arachidonic acid. The 15-HETE product was purified as the methyl ester, and the 15-HETE enantiomers were resolved on a Chiralpak AD column (25 × 0.46 cm) eluted with hexane/ethanol (100/2, by volume) at 1 ml/min flow rate. Fractions of 0.5 min were collected and counted for 3H (A) and 14C (B). Panel A, analysis of radiolabeled 15-HETE from 13-pro-S-3H substrate. Panel B, analysis of 15-HETE from 13-pro-R-3H substrate. The counts shown are corrected for channel crossover.

Analysis of COX-2 Reactions—Products were initially purified by reversed phase HPLC using a Beckman Ultrasphere ODS 5-μm column (25 × 0.46 cm) and a solvent of hexane/isopropl alcohol (80/20/0.01, by volume). Unlabeled standards (0.5–1 μg) were added prior to chromatography to facilitate UV detection of the peaks. The 15-HETE was methylated and further purified by SP-HPLC using an Alttech Econosil Silica 5-µm column (25 × 0.46 cm) and a solvent of hexane/isopropl alcohol (100/2, by volume). Chiral phase HPLC was performed on a 25 × 0.46-cm Chiralpak AD column (Chiral Technologies, Exton, PA) using hexane/ethanol (100/2, by volume) as the mobile phase. On this system, the 15-HETE methyl esters were well resolved with retention times of ~10 (15R) and ~15 (15S), respectively (1 ml/min flow rate). Fractions of 30 s were collected across the eluting peaks, evaporated to dryness, mixed with scintillant, and counted for 2 h each using a Packard Tri-Carb 1900 TR liquid scintillation analyzer to define the 3H/14C ratios of the base line and the chromatographic peaks. For liquid scintillation counting, the retention channel was set at 0–20 keV and the 14C channel at 20–156 keV. This resulted in a channel crossover of 33.5% of 14C into 3H, whereas the 14C crossover into the tritium channel was below 0.5%. The values given in Fig. 3 and Table I are corrected for channel crossover.

Unreacted arachidonic acid was collected from RP-HPLC and further purified by argentation chromatography (14) using a SCX column (25 × 0.46 cm, from Analytichem Int.) in silver form with hexane/isopropl alcohol/H2O/acetic acid (40/60/3/0.1, by volume) at 0.5 ml/min as mobile phase. Again, fractions of 30 s were collected across the eluting peaks, evaporated in the counting vials, and analyzed as described above.

Prostaglandins were collected from the initial RP-HPLC as a pooled fraction and separated on a second RP-HPLC system using a Waters Symmetry C18 5-μm column (25 × 0.46 cm) eluted with acetonitrile/H2O/acetic acid (35/65/0.1, by volume) at 1 ml/min flow rate. PGE2, which was the main prostaglandin product, was further purified by SP-HPLC on an Alttech Econosil Silica 5-µm column (hexane/isopropl alcohol/acetic acid, 90/10/1, by volume). The PGE2 was collected as a single peak from SP-HPLC, and an aliquot was counted to determine the 3H/14C ratio.
Mechanism of 15R-HETE Synthesis by Acetylated COX-2

Retention of tritium in the conversion of stereospecifically labeled arachidonic acids by acetylated and native COX-2

Acetylated (aspirin-treated) and native COX-2 were incubated with [3-14C; 13-pro-S-3H]- and [3-14C; 13-pro-R-3H]arachidonic acids as described under "Experimental Procedures." Products and recovered unreacted arachidonic acid were purified through at least two steps of HPLC, and the \(^{3}H/^{14}C\) ratios were determined as described (under "Experimental Procedures"); % tritium retention was obtained by comparison of the \(^{3}H/^{14}C\) ratios to the initial value for the arachidonic acid substrate (designated as 100%). Values given are corrected for channel crossover. The % conversion of arachidonic acid was not measured in these incubations, but from pilot experiments was estimated as 30 and 55% with 150 and 450 nM acetylated COX-2, respectively, and 70 and >95% with 0.5 and 1.8 \(\mu M\) native enzyme, respectively. In the latter incubation, the nearly complete conversion resulted in a tritium retention of 2700% in the unreacted arachidonic acid, reflecting the primary isotope effect on 13-pro-S-H abstraction (1).

Table I

| Cyclooxygenase preparation | [13-pro-S-3H]C20:4a6 | % Tritium retention | [13-pro-R-3H]C20:4a6 | % Tritium retention |
|----------------------------|----------------------|--------------------|----------------------|--------------------|
| Acetylated COX-2           |                      |                    |                      |                    |
| 450 nM                     | 3                    | 103                | 108                  | 96                 |
| 450 nM                     | 6                    | 120                | 147                  | 123                |
| 150 nM                     | 9                    | 98                 | 121                  | 100                |
| Native COX-2               |                      |                    |                      |                    |
| PGE\(_2\)                  | 3                    | 152                | 99                   | 81                 |
| 500 nM                     | 8                    | 2700               | 104                  | 120                |

Fig. 4. Illustration of reverse orientation of arachidonic acid in the acetylated COX-2 active site (A) and redirection of approach of molecular oxygen in acetylated COX-2 (B). Panel A, the top structure shows the usual cyclooxygenase reaction with pro-S hydrogen abstraction at C-13 of arachidonic acid and oxygenation in the 11R configuration. Immediately below is shown substrate in reversed orientation; this might explain 15R-HETE synthesis but is associated with pro-R hydrogen abstraction at C-13. Panel B, approach of molecular oxygen from above or below changes the configuration at C-15.

RESULTS

We used incubations with \([^{14}C]\)arachidonic acid to establish the appropriate enzyme and substrate concentrations for the subsequent experiments with stereospecifically labeled arachidonic acids. Fig. 2 shows the metabolism of \([^{14}C]\)arachidonic acid by native COX-2 and the effects of aspirin treatment. The native enzyme forms prostaglandins as the major polar products, together with HHT and minor amounts of 15-HETE and 11-HETE (Fig. 2A). Aspirin treatment of COX-2 blocked prostaglandin synthesis and the associated formation of HHT and 11-HETE; as expected, 15R-HETE was recovered as the major product (Fig. 2B) (10–12). Under the conditions of our incubations, we found that the peroxidase activity of the enzyme was not sufficient to reduce fully the 15R-HPETE to 15R-HETE. This resulted in the appearance of polar derivatives of the 15-hydroperoxide on the chromatograms (not shown). To eliminate these products we treated the samples with SnCl\(_2\) prior to extraction. This reduction step ensured that no secondary products were formed upon HPETE degradation that might have an influence on the isotopic composition of the products.

Following incubation with the stereospecifically labeled [13-\(^{3}H\)]arachidonic acids (admixed with [3-\(^{14}C\)]arachidonic acid as internal standard), the major products were purified through at least two steps of HPLC (see under "Experimental Procedures"), and the \(^{3}H/^{14}C\) ratios were determined by liquid scintillation counting. In the case of 15-HETE from the aspirin-treated COX-2 incubations, the final HPLC step was carried out on a chiral column and fractions were collected across the peaks, Fig. 3. By using [13-pro-S-\(^{3}H\)]arachidonic acid substrate (panel A) the main peak of 15R-HETE is comprised of \(^{14}C\) with very little retention of \(^{3}H\) label. This shows that the pro-S hydrogen is removed from C-13 during conversion of arachidonic acid to 15R-HETE. From [13-pro-R-\(^{3}H\)]arachidonic acid (panel B), it is clear that the tritium is retained in the 15R-HETE. The results from three separate experiments are given in Table I. The pro-S tritium was retained only 3–9% in the 15R-HETE, whereas the pro-R tritium showed 108–147% retention. Table I also summarizes the results of incubations with native COX-2 (not acetylated). The tritium retentions in the major cyclooxygenase product, PGH\(_2\) (measured as PGE\(_2\)), indicate removal of the pro-S hydrogen during catalysis and, correspondingly, retention of the pro-R radiolabel.
DISCUSSION

One of the main interests in conducting this study relates to potential insights into substrate binding in the cyclooxygenase active site. From x-ray structural analyses, the location and overall topologies of the substrate access channel and the binding site for cyclooxygenase catalysis are identified (6–9). There are also mutational analyses and modeling data on the binding of arachidonic acid (13, 15). There is not yet data on the precise conformation of arachidonic acid nor an explanation for the synthesis of 15R-HETE by aspirin-treated COX-2.

By using stereospecifically labeled arachidonic acid substrates, we established that the hydrogen abstraction associated with formation of 15R-HETE by aspirin-treated COX-2 is identical to the hydrogen abstraction in prostaglandin biosynthesis. This is surprising for a couple of reasons. First, prostaglandins and 15R-HETE have opposite configurations of the 15-hydroxyl, and accordingly, there is some fundamentally different stereochemistry involved in the two reactions. Second, the stereochemical relationship between hydrogen removal and the 15R-oxygenation would appear to contradict the antarafacial rule observed in all other cyclooxygenase and lipoxygenase reactions. This point is discussed later.

We considered three possible models to equate the stereochemistry of hydrogen abstraction with the stereochemistry of the 15R-HETE product. Before the results of the present study were in hand, a theoretical explanation of 15R-HETE synthesis could have involved a reversed orientation of substrate in the active site. This phenomenon is well preceded in lipoxygenase catalysis (e.g. Refs. 16 and 17). Assuming no other changes in the oxygenation machinery, a “head-to-tail” reorientation of arachidonic acid would lead to formation of 15R-HETE at the position that yields 11R-HETE in the normal alignment (Fig. 4, panel A, top). This model, however, predicts removal of the pro-R hydrogen at C-13 during catalysis. This is opposite to the observed experimental result, and thus this first possibility can now be dismissed.

A potential explanation that equates abstraction of the 13-pro-S hydrogen with synthesis of 15R-HETE entails approach of molecular oxygen from the opposite side of the substrate (Fig. 4, panel B) (11). The control of molecular oxygen in the two oxygenations in cyclooxygenase catalysis, assuming there is any active control, is not understood. Therefore, based on our present understanding it could be argued that we cannot dismiss the possibility of its reversal by acetylation of Ser-530. Notwithstanding this caveat, it remains true that there are no rational explanations for how acetylation of Ser-530 could lead to a change in the direction of approach of molecular oxygen. Furthermore, if acetylation of Ser-530 interferes only with the movement of molecular oxygen, it is even less clear how this could interfere with the initial cyclooxygenase reaction in the usual position at C-11. On a number of grounds, therefore, we consider this explanation unlikely.

A third model to account for the experimental results predicts that acetylation of Ser-530 constricts the available space normally occupied by the omega end of the arachidonic acid carbon chain. In the native enzyme (Fig. 5, panel A) a radical on Tyr-385 initiates the cyclooxygenase reaction by abstraction of the pro-S hydrogen at C-13 of arachidonic acid (18). This is followed by two successive oxygenations involving approach of molecular oxygen from behind the plane of view to react initially in the 11R configuration and subsequently 15S. In the acetylated enzyme (Fig. 5, panel B), the bulky acetyl group has blocked the normal arachidonic acid conformation and forced a twisting over of the tail end of the carbon chain. The approach of molecular oxygen in the normal way will now lead to reaction in the 15R configuration. It is also apparent that C-11 might no longer be accessible for oxygen attack as it is “protected” or shielded by the arachidonic acid omega carbon chain, and, as a consequence of the lack of oxygenation at C-11, prostaglandin synthesis is blocked. This model, therefore, can account for the production of 15R-HETE as the sole product of aspirin-treated COX-2.

In characterizing oxygenase reactions, the relative stereochemistry of hydrogen abstraction and oxygen insertion can be described as suprafacial (the two occur on the same face of the substrate) or antarafacial. When the term antarafacial is applied to cyclooxygenase and lipoxygenase reactions, it is assumed that the 1,4-pentadiene is in a planar conformation with the two cis double bonds lying in parallel. From this viewpoint, all lipoxygenase and cyclooxygenase oxygenations, except for the 15R-HETE synthesis studied here, are antarafacial. This has proved a useful concept for unifying the diverse reactions of these families of oxygenase. The terminology is of more questionable value when the pentadiene is viewed in different conformations, for example with one double bond twisted over, as in the model of arachidonic acid in Fig. 5B. In examples like this this terminology loses its utility and is probably best avoided.

As a final point, there is one more line of argument that supports the realignment explanation for the synthesis of 15R-HETE in acetylated COX-2. This relates to the known differences in the active sites of COX-1 and COX-2, the very differences that contribute to the selectivity of COX-2 inhibitors. Comparison of the crystal structures of the two enzymes indicate that COX-2 has more available space in the active site (8, 9), including in the central channel where substrate binds (8). If a change in approach of molecular oxygen were the only significant consequence of acetylation of the COX enzymes, it is unclear why COX-2 retains the ability to synthesize 15R-HETE while the oxygenase activity of COX-1 is completely blocked. Considering instead that the COX-2 active site has more available space, this fits with a model that predicts movement of the omega carbon chain in acetylated COX-2, a realignment that is not physically possible in the more restricted active site of acetylated COX-1.

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