The Binding of Arachidonic Acid in the Cyclooxygenase Active Site of Mouse Prostaglandin Endoperoxide Synthase-2 (COX-2)

A PUTATIVE L-SHAPED BINDING CONFORMATION UTILIZING THE TOP CHANNEL REGION*

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Abstract

The chemical mandates for arachidonic acid conversion to prostaglandin G₂ within the cyclooxygenase (COX) active site predict that the substrate will orient in a kinked or L-shaped conformation. Molecular modeling of arachidonic acid in sheep COX-1 confirms that this L-shaped conformation is possible, with the carboxylate moiety binding to Arg-120 and the ω-end positioned above Ser-530 in a region termed the top channel. Mutations of Gly-533 to valine or leucine in the top channel of mCOX-2 abolished the conversion of arachidonic acid to prostaglandin G₂, presumably because of a steric clash between the ω-end of the substrate and the introduced side chains. A smaller G533A mutant retained partial COX activity. The loss of COX activity with these mutants was not the result of reduced peroxidase activity, because the activity of all mutants was equivalent to the wild-type enzyme and the addition of exogenous peroxide did not restore full COX activity to any of the mutants. However, the Gly-533 mutants were able to oxidize the carbon 18 fatty acid substrates linolenic acid and stearidonic acid, which contain an allylic carbon at the ω-5 position. In contrast, linoleic acid, which is like arachidonic acid in that its most ω-proximal allylic carbon is ω-8 position, was not oxidized by the Gly-533 mutants. Finally, the ability of Gly-533 mutants to efficiently process ω-5 allylic substrates suggests that the top channel does not serve as a product exit route indicating that oxygenated substrate diffuses from the cyclooxygenase active site in a membrane proximal direction.

Prostaglandin endoperoxide synthesis has two activities that are required for the production of PGH₂, the essential precursor of prostaglandins, thromboxane, and prostacyclin (1). The first activity catalyzes the oxygenation and cyclization of arachidonic acid within the cyclooxygenase (COX) active site to produce PGG₂. PGG₂ then exits the cyclooxygenase active site and undergoes a two-electron reduction, yielding PGH₂ at the peroxidase active site (2). Two isoforms of COX exist, COX-1 and COX-2, that effect the same enzymatic reactions (3). They are approximately 60% identical in sequence and are highly homologous in both active site regions. Not surprisingly, their three-dimensional structures are nearly superimposable (4–6). COX-1 and COX-2 are mediators of numerous physiological and pathological responses, and therefore considerable effort has been devoted to developing selective COX inhibitors. This is especially true of COX-2, which is a significant contributor to inflammation, hyperalgesia, and cancer (7). Structural analysis of COX-inhibitor complexes has provided a detailed understanding of their interaction with the enzyme and insight into the mechanism of isoform selectivity (4–6, 8).

A less detailed picture is available for the interaction of the substrate arachidonic acid with the proteins. Crystal structures of COX-arachidonic acid complexes have not been reported, and so most of the available information has been developed by employing site-directed mutagenesis. It is generally agreed that Arg-120 is the ion pairs or hydrogen bonds to the carboxylic group of the fatty acid (9–12) and that Tyr-385 removes the 13-pro-S-hydrogen in the first step of oxygenation (13, 14). However, the orientation of the rest of the substrate molecule, particularly the ω-end, is uncertain.

We have approached the problem of arachidonic acid-COX interaction by attempting to match the chemical mandates of the cyclooxygenase reaction to complementary regions on the protein. COX catalyzes the conversion of an achiral molecule into a product with five chiral centers (15). Noteworthy is the generation of the endoperoxide ring with the trans-substituted alkyl side chains. This stereochemistry is opposite to that observed in the auto-oxidation of arachidonic acid in solution in which the alkyl side chains are oriented cis (16). Thus, the enzyme must orientate the arachidonic acid molecule in the COX active site to facilitate the formation of the trans-substituted ring. Any model for COX-arachidonate binding must accommodate this stereochemical mandate. Some time ago it was predicted that the enzyme holds arachidonate in a kinked or L-shaped conformation to facilitate cyclization to form a trans-disubstituted dioxygenycycloheptane ring (17) (Fig. 1). Therefore, we modeled such a conformation into the COX-1 active site with the carboxylate adjacent to Arg-120 and the 13-pro-S-hydrogen adjacent to Tyr-385; this starting structure was then energy-minimized. The minimized conformation positioned the ω-end of arachidonate above Ser-530 projecting into a region that we term the top channel.

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1 The abbreviations used are: PG, prostaglandin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; COX, cyclooxygenase; mCOX-2, mouse cyclooxygenase-2; 11-(R)-HETE, 11-(R)-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid; 15-(R)-HETE, 15-(R)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; PPIX, protoporphyrin IX.

2 The numbering system used is in accord with that of COX-1.
the top channel was tested using site-directed mutagenesis of murine COX-2, which can be expressed at high levels in insect cells and purified to homogeneity. The results of the mutagenesis experiments are strongly supportive of a role for the top channel in binding the ω-end of arachidonic acid and are consistent with the predictions of the energy minimized model.

**EXPERIMENTAL PROCEDURES**

**Materials**—Linoleic acid (9Z,12Z-octadecadienoic acid), linolenic acid (9Z,12Z,15Z-octadecatrienoic acid), and stearidonic acid (6Z,9Z,11Z,14Z-eicosatetraenoic acid) and [14C]arachidonic acid were purchased from Nu-Check-Prep, Inc. and NEN Life Science Products, respectively. Electrophoresis and chromatography reagents were purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents were purchased from Sigma unless otherwise stated.

**Modeling**—All modeling of arachidonic acid and sheep COX-1 was performed using InsightII ( Biosym Technologies, San Diego, CA) in the manner described previously (18). Arachidonic acid was built using the Builder module and then positioned within the cyclooxygenase active site with the carboxylate group in close proximity to Arg-120 and Tyr-355. The arachidonate main chain was then oriented upward to optimally position the substrate in the sheep COX-1 active site. In the final model (Fig. 2), the carboxylic acid moieties of arachidonic acid are 3.0 and 3.1 Å from Arg-120 and Tyr-355, respectively, and the 13-ω-end of arachidonic acid protrudes into the solvent-accessible cavity toward Gly-533. Carbon 20 resides 3.3 Å from the hydrogen adjacent to Tyr-385. The ω-end of arachidonate was then placed above Ser-530 and Leu-534 and project into the solvent-accessible cavity toward Gly-533. Maintenance of all cis double bonds and positioning of the 13-pro-S-hydrogen in close proximity to Tyr-385.

**Mutation Construction**—Site-directed mutagenesis was performed on a mCOX-2 pBSI (+) vector (Stratagene, La Jolla, CA) using the Quick Change site-directed mutagenesis kit (Stratagene). Mutant containing regions were subcloned into the mCOX-2 pVL1393 baculovirus expression vector (PharMingen, San Diego, CA) using the Stul restriction site in mCOX-2 and the XhoI restriction site present in both the pBSI (+) and pVL1393 vectors. The subcloned region was fully sequenced to ensure that no accidental mutations were incorporated.

**Protein Expression and Purification**—Wild-type and mutant protein was expressed by homologous recombination of the mCOX-2-pVL1393 vector with the Baculogold vector (PharMingen) in SF-9 cells (Novagen, Madison, WI). After virus amplification, 4 liters of SF-9 cells (95–100% viable) were grown in TNM-FH medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% l-glutamine, and 0.1% (v/v) pluronic F68 and then infected with fresh viral stock. Upon reaching 65–70% viability, the 4-liter total volume was harvested by centrifugation at 2,500 rpm in a Sorvall RC5C at 4 °C and the pellet washed in ice-cold phosphate-buffered saline and recentrifuged. The final cell pellet was stored at −70 °C.

Purification of wild-type and mutant COXs were performed at 4 °C in a manner similar to that described previously (19). Frozen cells were resuspended to 30 × 10⁶ cells/ml in 80 mM Tris-HCl, 2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, and 0.1 mM diethyldithiocarbamic acid, pH 7.2. After centrifugation at 100,000 × g for 45 min, the pellet was resuspended using a Dounce homogenizer to a final volume of 72 ml. Solubilization of the COX protein from the membrane was initiated by the dropwise addition of 8 ml of 1% (w/v) CHAPS. After stirring for 1 h, the sample was centrifuged as described above and the supernatant removed and then dialyzed 4-fold by the addition of 20 mM Tris-HCl, 0.4% CHAPS, 0.1 mM EDTA, and 0.1 mM diethyldithiocarbamic acid, pH 8.0 (Buffer B). The diluted sample was then loaded onto a 25-ml Macro-prep High-Q ion exchange column equilibrated with Buffer B. COX enzyme was eluted with a linear gradient (500 ml) of increasing KCl to 0.3 M. An analytical 7.5% SDS-polyacrylamide gel electrophoresis was run of candidate COX-containing fractions to determine the fractions containing COX enzyme activity. Fractions containing COX enzyme, as determined by SDS-polyacrylamide gel electrophoresis analysis (as described above), were concentrated to approximately 2 mg/ml and stored at −70 °C. The purity of wild-type and mutant COX-2 proteins was evaluated by analysis of a Coomassie-stained 7.5% SDS-polyacrylamide gel using an E-C Apparatus Model EC910 scanning densitometer.

**Cyclooxygenase Activity**—Oxygen consumption was measured with a Gilson Model 5/6 oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) fitted with a Clark electrode and a thermostatted cuvette set to 37 °C in a 100 mM Tris-HCl, 500 μM phenol, pH 8.0, buffer. The rate and magnitude of oxygen uptake were determined in the manner previously described (20).

**Peroxidase Assays**—The peroxidase activity of all purified proteins was measured using the guaiacol peroxidase assay. Purified wild-type mCOX-2 or mCOX-2 mutants were suspended in 1 ml of 118 mM Tris-HCl, pH 8.0, at a final concentration of 100 nM. Guaiacol at 500 μM was added, and the reaction mixtures were frozen in liquid nitrogen or heated to 70 °C. The purity of wild-type and mutant COX-2 proteins was evaluated by analysis of a Coomassie-stained 7.5% SDS-polyacrylamide gel using an E-C Apparatus Model EC910 scanning densitometer.

**Cyclooxygenase Product Assays**—Cyclooxygenase product assays were performed with purified protein reconstituted with 2 eq of heme. Reactions were initiated by the addition of 100 μM [14C]arachidonic acid. Conditions such as the concentration of phenol, supplementation with H₂O₂, and time and were varied and are indicated in the figure captions. All assays were terminated and analyzed by thin layer chromatography in the manner described previously (21).

**RESULTS**

**Modeling**—Arachidonate was anchored at the carboxyl end through interactions with Arg-120 and Tyr-355 and by positioning the 13-pro-S-hydrogen adjacent to Tyr-385. The ω-end was placed into the region we term the top channel. Several rounds of energy minimization and refinement were carried out to optimally position the substrate in the sheep COX-1 active site. In the final model (Fig. 2), the carboxylic acid moieties of arachidonic acid are 3.0 and 3.1 Å from Arg-120 and Tyr-355, respectively, and the 13-pro-S-hydrogen is 2.4 Å from the hydroxyl of Tyr-385. The ω-end of arachidonate protrudes into the top channel region, and although the opening to this channel is narrow, there is sufficient room for carbons 17–20 to reside above Ser-530 and Leu-534 and project into the solvent-accessible cavity toward Gly-533. Carbon 20 resides 3.3 Å from the α carbon of Gly-533.

**Mutant COX-2 Characterization**—To test this hypothesis, we constructed a series of site-directed mutants at Gly-533. This...
residue is conserved in all COX sequences, and we anticipated that increasing the steric bulk at this position would reduce arachidonate binding. Both wild-type and mutant COX-2 cDNAs were expressed in insect cells from baculovirus vectors, and recombinant proteins were purified by ion exchange and gel filtration chromatography as described under “Experimental Procedures.” All of the purified proteins were shown by densitometric scanning of a 7.5% SDS-polyacrylamide gel to be equal to or greater than 80% pure (Table I).

Table I lists both the peroxidase activity and the cyclooxygenase activity of the three Gly-533 mutants. The similarities of the peroxidase activities to that of wild-type COX-2 demonstrate that the mutations did not introduce gross structural perturbations. However, the cyclooxygenase activities of all three mutants were affected significantly. The G533L and G533V mutants were unable to convert arachidonate to PGG2, whereas the G533A mutant had a much slower initial rate for substrate conversion and demonstrated just 26% of wild-type total COX-2 activity.

One possibility that could explain the low turnover rate observed with G533A could be a decreased activation of cyclooxygenase catalysis. PGG2 released from the cyclooxygenase active site is converted to PGH2 at the peroxidase active site with concomitant formation of a heme-oxo complex (22). This complex oxidizes Tyr-385 and generates the catalytically active tyrosyl radical. As the G533A substitution compromises conversion of arachidonate to PGG2, this lower activity could be further pronounced because of less PGG2 available to maximize all the COX molecules in solution. To address the concern of insufficient peroxide activation, three different approaches were employed: 1) lowering the concentration of reducing substrate (phenol) in the assay buffer to slow down the reduction of exogenous peroxides and cyclooxygenase-synthesized fatty acid hydroperoxides; (2) adding H2O2 to generate higher oxidation states of the peroxide; and (3) adding enzyme-synthesized fatty acid hydroperoxide as an activator.

Reducing the phenol concentration from 500 to 100 μM resulted in an increase in cyclooxygenase activity of the G533A mutant from 5.5 to 18.9% converted substrate (Fig. 3A). Likewise, the addition of 15 μM H2O2 increased the conversion of arachidonate from 3.4 to 22% (Fig. 3B). However, changing these conditions did not restore cyclooxygenase activity to the level observed with an equivalent amount of wild-type mCOX-2 nor did it restore any activity to either G533V or G533L. To evaluate whether PGG2 could enhance G533A turnover, G533A mCOX-2 was incubated with a 13-fold lower concentration of Mn-PPIX reconstituted ovine COX-1. Mn-PPIX reconstituted ovine COX-1 has full cyclooxygenase activity but only 0.8% of the peroxidase activity of Fe-PPIX reconstituted COX-1 (23, 24). Thus, G533A mCOX-2 should be able to use the COX-1-derived PGG2 to increase the rate of tyrosyl radical formation and cyclooxygenase activity. After subtracting the cyclooxygenase activity resulting from COX-1 turnover, it was found that the G533A activity was equivalent to that observed when G533A was incubated with 15 μM H2O2 as an activator. These results suggest that the reduced cyclooxygenase activity of the G533A mutant leads to a slower rate of auto-activation. However, even when maximally activated, the cyclooxygenase activity of this mutant is reduced by 80% compared with the wild-type enzyme, and the G533V and G533L mutants are completely inactive.

Analysis of the arachidonate/sheep COX-1 model suggests that the most likely reason for the reduced activity is steric hindrance between arachidonate carbon 20 and the introduced side chains at position 533. Therefore, the Gly-533 mutants were compared with wild-type enzyme for their ability to metabolize two fatty acids that are substrates for COX-2 but contain an abstractable hydrogen closer to the ω-ω (ω-5 po-
results of experiments on the cyclooxygenase activity of G533A mCOX-2, G533A (44 nM) was incubated in 100 mM Tris-HCl, pH 8, with the indicated concentration of phenol and 50 µM 14C-arachidonic acid for 5 min at 37 °C; and for B, the reactions were acidified with cold termination solution, run on silica thin-layer chromatographic plates, and scanned for radioactive products in the manner indicated under “Experimental Procedures.” Each data point is the average of three determinations ± S.E.

H2O2 but were then supplemented with H2O2 after 2 min to complete the reaction (Tables III). Comparison of wild-type mCOX-2 treated with or without 15 µM H2O2 (Tables II and III) showed that the addition of H2O2 had little effect on either the rate or the total substrate conversion. The Gly-533 mutants, in contrast, showed much more dependence on H2O2, as both the rate and amount of substrate converted were reduced in its absence. The addition of 15 µM H2O2 to the reaction was able to reactivate substrate conversion for the Gly-533 mutants.

**DISCUSSION**

We propose a model for the binding of arachidonic acid in the cyclooxygenase active site that is consistent with the chemical mandates for its oxidation to PGG2 and with existing information on its interaction with individual protein residues. The carboxylate of arachidonate is anchored to Arg-120 and Tyr-355 by ionic and hydrogen bonding interactions, respectively (Fig. 2). The substrate backbone then projects upward into the active pocket of the cyclooxygenase active site where it bends around the 9,10-double bond into an L-shape. Carbon 13 is positioned under Tyr-385 with the 13-hydroxyl group. Finally, the v-n-13-hydrogen is re-orientation of this structural model suggests that the individual reactions can occur with minimal motion of the bound intermediates. Furthermore, the model predicts the generation of PGG2 with all five stereocenters in the correct absolute configuration, because once the arachidonate is bound as indicated in Fig. 2, the only available space through which O2 can approach the radical intermediates is through the center of the active site channel. Thus, O2 approaches the bound fatty acid from the opposite side from which the 13-pro-S-hydrogen is removed. This antarafacial relationship is consistent with the stereochemistry of PGG2.

The key feature of our model is that the ω-end of arachidonate projects into an area of the protein we term the top channel, which is located above Ser-530 and Leu-534. There are two major pieces of experimental information consistent with this hypothesis. The first is that introduction of steric bulk at position 533 by site-directed mutagenesis significantly reduces or completely abolishes the ability of the mutant COX-2s to oxidize arachidonic acid. The loss of oxygenase activity is not due to a major structural change in the proteins, because the peroxidase activity of each of the position 533 mutants is identical to that of wild-type enzyme. Likewise, the loss of activity is not due to an inability of the mutant proteins to activate the cyclooxygenase activity, although activity is stimulated to some extent by addition of H2O2. The second piece of experimental information consistent with a role for the top channel in arachidonate binding is that all of the position 533 mutants are able to oxidize unsaturated fatty acid substrates that contain three less carbons at their ω-end than arachidonate relative to the position of the hydroxyl abstracted by the tyrosyl radical of

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*Results virtually identical to those seen in Table III with linolenic acid were obtained with stearidonic acid.*
Tyr-385. By contrast, the mutants are unable to oxidize linoleic acid; oxidation of stearidonic acid is that the top channel is part of an exit route for products.

Our model is consistent with the chemistry of the production of PGG$_2$ from arachidonic acid and with all the currently available site-directed mutagenesis results. Thus, it is likely that this is the conformation by which COX enzymes convert arachidonic acid to its major enzymatic product. This conclusion does not rule out the possibility that arachidonate binds in the cyclooxygenase active site in alternate conformations. For example, arachidonate is oxidated to a series of hydroxy acids by cyclooxygenase including 11- (S)-HETE, 15- (S)-HETE, and 15- (R)-HETE (15, 26). The production of 11- (S)-HETE and 15- (S)-HETE has been assumed to result from O$_2$ trapping of carbon radicals produced following removal of the 13-pro-S-hydrogen. However, 15- (R)-HETE is produced by aspirin-acetylated COX-2 and is likely to arise from an alternate conformation of arachidonic acid (26). Indeed, Xiao et al. (27) have recently shown that 15- (R)-HETE also can be made by unacetylated COX-2 at high concentrations of arachidonic acid. Thus, 15- (R)-HETE appears to represent an alternate product that results from a less favored substrate conformation.

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### Table II

**Activity of H$_2$O$_2$-activated wild-type and Gly-533 mutants with different fatty acid substrates**

| Mutant      | Oxygen consumption after H$_2$O$_2$ (mol/min/mg) |
|-------------|-----------------------------------------------|
| arachidonic | G533A  16.7 ± 0.3 | G533V  19.7 ± 0.2 | G533L  21.2 ± 0.3 |
| linoleic    | 9.6 ± 0.2 | 6.7 ± 0.6 | 6.6 ± 0.3 |
| O$_2$ consumed | 50.7 ± 0.2 | 17.0 ± 0.2 | 25.0 ± 0.5 |
| stearidonic | 5.3 ± 0.6 | 2.9 ± 0.2 | 2.7 ± 0.1 |
| O$_2$ consumed | 0.6 ± 0.1 | 1.3 ± 0.1 | 0.6 ± 0.1 |

### Table III

**Activity of wild-type and Gly-533 mutants with linolenic acid before and after H$_2$O$_2$ addition**

| Mutant      | Oxygen consumption with linolenic acid (mol/min/mg) | Rate with linolenic acid after H$_2$O$_2$ (mol/min/mg) |
|-------------|-----------------------------------------------------|-----------------------------------------------------|
| Wild type   | 12.9 ± 0.2 | 6.1 ± 0.3 |
| G533A       | 5.8 ± 0.4 | 1.9 ± 0.2 |
| G533V       | 2.5 ± 0.0 | 1.0 ± 0.2 |
| G533L       | 0.0 ± 0.0 | 6.8 ± 0.3 |
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