Cyclooxygenase mechanisms

Lawrence J Marnett

Several advances have occurred in the past year in our understanding of cyclooxygenase catalysis. The role of specific heme oxidation states in the formation of catalytically competent tyrosyl radicals has been defined; the identity of physiological hydroperoxide activators has been established; and the participation of individual amino acids in substrate binding and oxygenation has been elucidated.

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Abbreviations
COX cyclooxygenase
PG prostaglandin

Introduction

Cyclooxygenases (COXs) catalyze the committed step in the conversion of arachidonic acid to prostaglandins and thromboxane. They oxygenate arachidonic acid to the hydroperoxy endoperoxide PGG_2 (prostaglandin G_2), followed by reduction of PGG_2 to the alcohol PGH_2 (Figure 1). PGH_2 is converted by isomerases to prostaglandins and thromboxane, which exert numerous physiological and pathophysiological effects. Thus, COX enzymes play a key role in the biosynthesis of a family of important bioactive lipids. But it is the interesting chemistry which they catalyze that is the focus of this review. Recent advances in the mechanism of arachidonic acid oxygenation, the identity of the protein oxidant, the pathway of enzyme activation, and the nature of enzyme–substrate interactions will be described. Amino acid designations are given based on the COX-1 numbering system.

Mechanism of arachidonate oxygenation

The conversion of arachidonic acid to PGG_2 can be formulated as a series of radical reactions analogous to those of polyunsaturated fatty acid autoxidation (Figure 2) [1]. The 13-*pro(S)*-hydrogen is removed and O_2 traps the incipient pentadienyl radical at C-11. The 11-peroxyl radical cyclizes at C-9 and the carbon-centered radical generated at C-8 cyclizes at C-12, producing the endoperoxide. The allylic radical generated is trapped by O_2 at C-15 to form the 15-*(S)*-peroxyl radical; this radical is then reduced to PGG_2. Several pieces of experimental evidence support this mechanism: firstly, a significant kinetic isotope effect is observed for the removal of the 13-*pro*(*S*)-hydrogen [2]; secondly, carbon-centered radicals are trapped during catalysis [3]; and thirdly, minor oxidation products are formed that arise by oxygen trapping of an allylic radical intermediate at positions 13 and 15 [4,5]. A variation of the mechanism in Figure 2 in which the 13-*pro*(*S*)-hydrogen is removed as a proton and the incipient carbanion is oxidized to a radical is theoretically possible. However, oxygenation of 10,10-difluoroarachidonic acid to 11-(*S*)-hydroxyeicosa-5,8,12,14-tetraenoic acid is inconsistent with the occurrence of a carbanion intermediate because the latter would rapidly eliminate fluoride to form a conjugated diene [6]. The absence of endoperoxide-containing products derived from 10,10-difluoroarachidonic acid has been suggested to indicate the importance of a C-10 carbocation in PGG_2 synthesis [7]. However, the proposed cationic mechanism postulates that endoperoxide formation precedes removal of the 13-*pro*(*S*)-hydrogen [7]. This is inconsistent with the results of isotopic labeling experiments of arachidonic acid oxygenation [2].

Identity of the protein oxidant

The oxidant that removes the 13-*pro*(*S*) hydrogen appears to be a tyrosyl radical derived from Tyr385 (Figure 2) [8]. This residue is interposed between the heme prosthetic group and the cyclooxygenase active site and is ideally positioned to interact with a bound fatty acid molecule [9,10,11••]. Transient tyrosyl radicals are detected during cyclooxygenase catalysis and they oxidize arachidonic acid to carbon-centered radicals [12••]. It has been difficult to assign the identity of the tyrosyl radicals based solely on electron paramagnetic

Figure 1

The conversion of arachidonic acid to PGH_2. COX catalyzes the oxidation of arachidonic acid to PGG_2, followed by reduction of PGG_2 to the alcohol PGH_2. This is then converted by isomerases to prostaglandins and thromboxane, which exert numerous physiological and pathophysiological effects. PER, peroxidase.

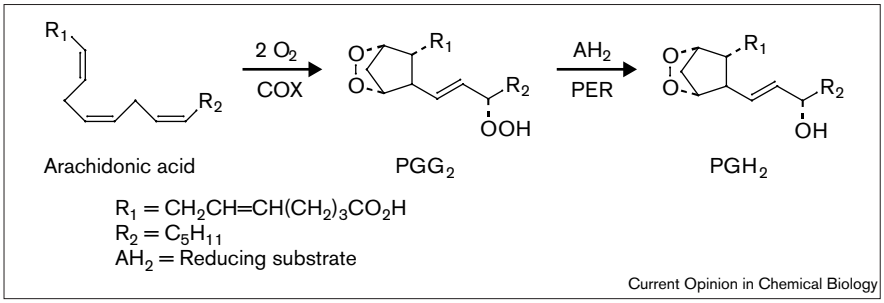
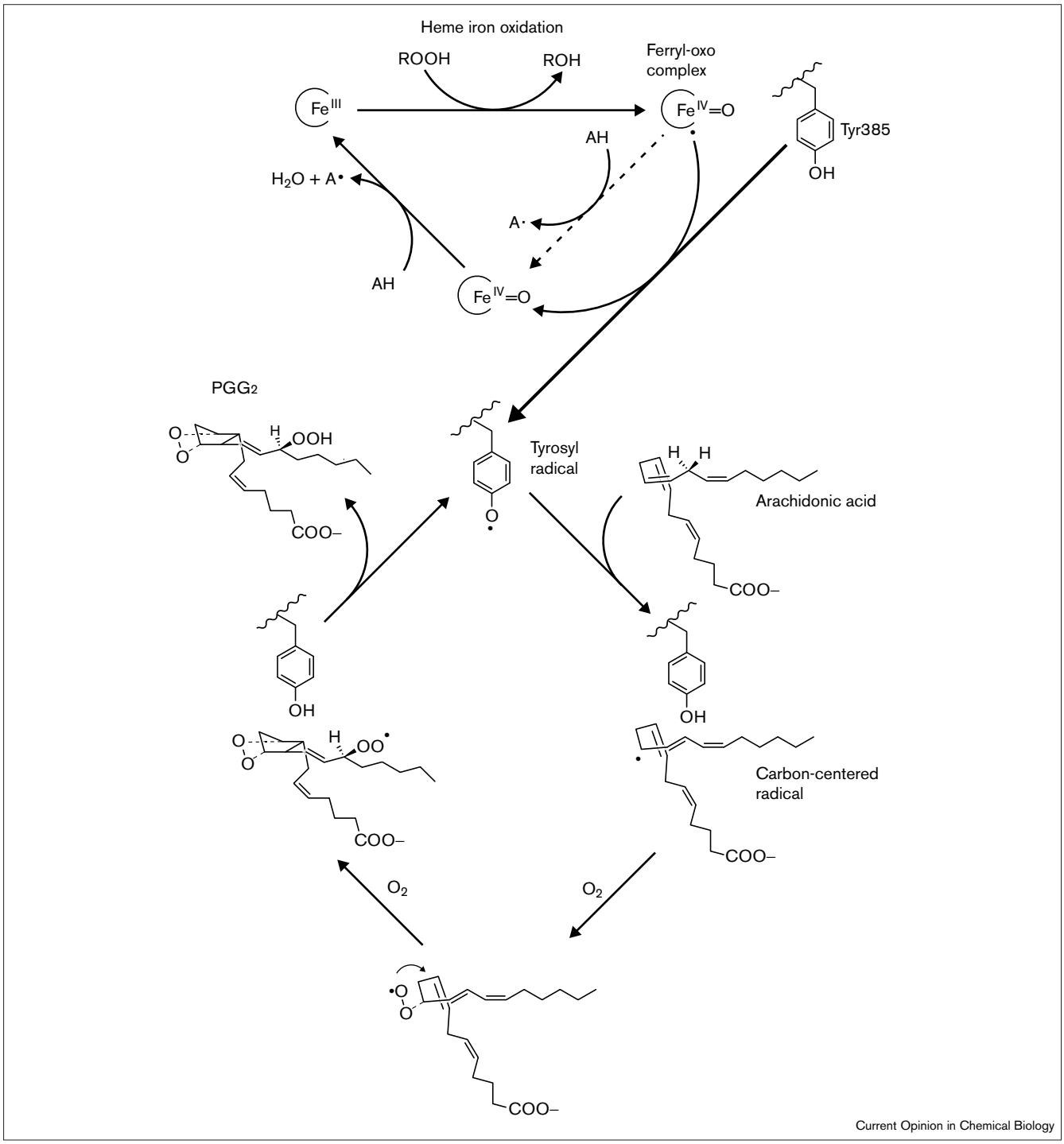


Figure 2



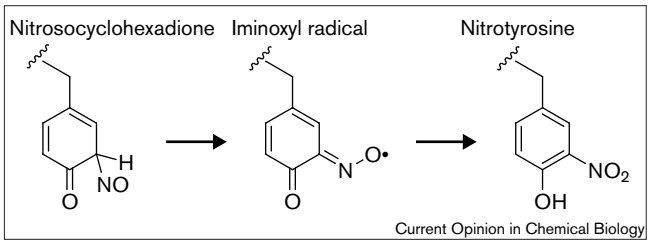
Overall mechanism of COX activation and catalysis. A hydroperoxide oxidizes the heme prosthetic group to a ferryl-oxo derivative that can be reduced in the first step of the peroxidase catalytic cycle or can oxidize Tyr385 to a tyrosyl radical (upper half of figure). The tyrosyl radical then oxidizes the 13-*pro*(*S*) hydrogen of arachidonic acid to initiate the cyclooxygenase catalytic cycle (lower half of figure).

resonance (EPR) spectroscopy [13,14]. Multiple hyperfine splitting patterns are observed that arise from rotational isomers of tyrosyl radicals and possibly tyrosyl radicals derived from different amino acids [15,16•]. Site-directed mutation of Tyr385 to Phe abolishes cyclooxygenase activity but does not eliminate radical production following reaction with a hydroperoxide (see below) [17]. Because there are several tyrosine residues at distances from the heme that are comparable to that of Tyr385, another tyrosine residue may be oxidized when

Tyr385 is absent. Interestingly, the Tyr385Phe mutant enzyme does not oxidize arachidonic acid to carbon-centered radicals, even though it does produce tyrosyl radicals following treatment with a hydroperoxide [12••].

The enzymatically generated tyrosyl radical has been trapped by carrying out reactions of arachidonic acid and COX-1 in the presence of NO donors [18]. NO quenches the tyrosyl radical signals, presumably by forming a nitrosocyclohexadienone. The nitrosocyclohexadienone is oxidized to an iminoxyl radical and ultimately to nitrotyrosine (Figure 3). Tryptic digestion and peptide mapping reveal the presence of a single nitrated peptide that contains a nitrotyrosine at the position in the sequence corresponding to Tyr385 [19••]. Formation of this nitrated peptide requires cyclooxygenase turnover in the presence of NO and is blocked by the cyclooxygenase inhibitor indomethacin.

Figure 3



Trapping of the enzymatically generated tyrosyl radical by carrying out reactions of arachidonic acid and COX-1 in the presence of NO donors. NO quenches the tyrosyl radical signals, presumably by forming a nitrosocyclohexadienone. The nitrosocyclohexadienone is oxidized to an iminoxyl radical and ultimately to nitrotyrosine.

Role of the heme

The Tyr385 tyrosyl radical is not present in resting enzyme so it must be generated in order to initiate cyclooxygenase catalysis. Reaction of fatty acid hydroperoxides or organic hydroperoxides with the heme prosthetic group generates a higher oxidation state of the heme that oxidizes Tyr385 (Figure 2) [20]. The higher oxidation state that oxidizes Tyr385 is the ferryl-oxo complex, which is the first intermediate in peroxidase catalysis (Compound I) [20,21]. Decay of the visible absorbance of Compound I coincides with production of the tyrosyl radical [22•].

Alterations in enzyme activity that reduce peroxidase activity introduce a lag phase in cyclooxygenase activation [23]. For example, mutations of the proximal histidine residue to tyrosine (His388Tyr) or the distal histidine to alanine (His207Ala) reduce peroxidase activity by 2 to 4 orders of magnitude and induce lag phases of 1 to 2 minutes for attainment of maximal cyclooxygenase activity following addition of arachidonic acid [21,24••]. This lag phase is eliminated by addition of exogenous hydroperoxides. The ability of a hydroperoxide to eliminate the lag phase correlates to its ability to serve as a peroxidase substrate [24••,25]. In the case of the distal histidine mutant (His207Ala), the lag phase also can be eliminated by adding large amounts of 2-methylimidazole to chemically reconstitute the peroxidase activity by providing a distal base to facilitate proton transfer during Compound I formation (Figure 4) [21].

Generation of Compound I by reaction of ferric enzyme with hydroperoxide establishes a thermodynamically favorable sequence of reactions to initiate cyclooxygenase catalysis. The redox potential for Compound I is estimated to be ~1 V by comparison with the analogous ferryl-oxo complex of horseradish peroxidase [26]. Reaction of Compound I with a tyrosine residue is exothermic ($E_o' = 0.9$ V) and so is the reaction of the tyrosyl radical with the doubly allylic hydrogens of a polyunsaturated fatty acid ($E_o = 0.6$ V) [27,28]. In contrast, the redox potentials for the resting ferric enzymes are −167 mV and −156 mV for COX-1 and COX-2, respectively, making direct oxidation of Tyr385 by ferric enzyme highly unfavorable thermodynamically [24••,29]. The low redox potentials of COX-1 and COX-2 are consistent with the observation that the resting enzymes are isolated in the ferric form and do not contain a spectroscopically detectable tyrosyl radical. Theoretically, it is possible that the endothermic nature of the oxidation of Tyr385 by ferric enzyme is circumvented by electron tunneling [30]. This may explain the activation of a derivative of COX-1 modified with bromoacetamido-indomethacin, which has no detectable peroxidase activity [31,32].

Figure 4

For the distal histidine mutant (His207Ala), the lag phase can be eliminated by adding large amounts of 2-methylimidazole to chemically reconstitute the peroxidase activity. The 2-methylimidazole acts as a distal base to facilitate proton transfer during Compound I formation. Compound I oxidizes Tyr385 to initiate cyclooxygenase catalysis (see Figure 2).

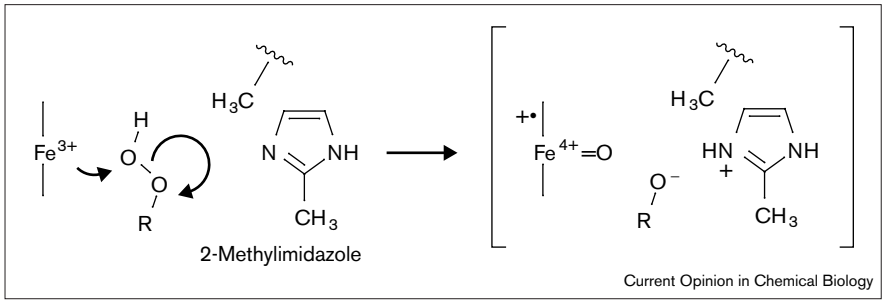
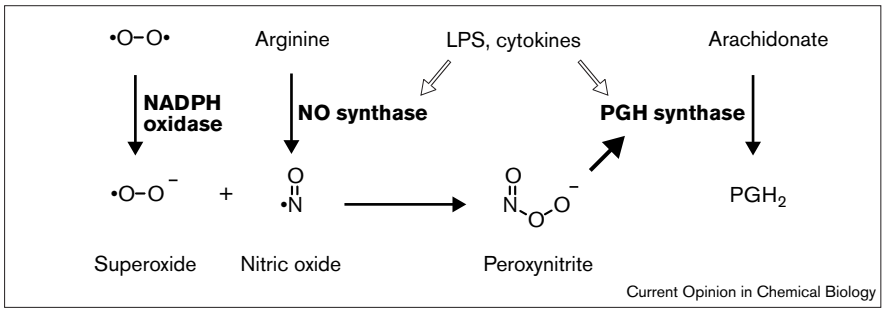


Figure 5



Biochemical linkage of nitric oxide biosynthesis with COX activation mediated by peroxynitrite. Hollow arrows represent stimulation of gene expression and enzyme synthesis. LPS, lipopolysaccharide.

Hydroperoxide activators

The activation of resting enzyme following addition of arachidonic acid *in vitro* is due to the presence of trace amounts of hydroperoxide in the fatty acid preparation. Activation is completely inhibited by addition of high concentrations of glutathione peroxidase and glutathione, which reduces fatty acid hydroperoxides [33–35]. Once the Tyr385 radical is generated, each enzyme molecule catalyzes several hundred cycles of arachidonic acid oxygenation. Although the tyrosyl radical is reduced to tyrosine when it oxidizes arachidonic acid, the radical is regenerated in the last step of each catalytic cycle by oxidation by the peroxyl radical precursor to PGG_2. There is occasional leakage of the peroxyl radical from the cyclooxygenase active site, which leaves the enzyme in a catalytically inactive form containing fully covalent Tyr385. Reactivation of cyclooxygenase activity requires reaction of the heme prosthetic group with another molecule of hydroperoxide. This explains the need for the continued presence of hydroperoxide in cyclooxygenase–arachidonic-acid reactions [36]. However, by and large, the cyclooxygenase catalytic cycle proceeds independently of the peroxidase catalytic cycle once Tyr385 is oxidized to a tyrosyl radical. This is supported by three pieces of evidence: the ability to isolate PGG_2 as the major product of arachidonic acid oxygenation [37,38]; detailed kinetic analyses consistent with independent turnover of the peroxidase and cyclooxygenase activities after activation [39]; and the ability of site-directed mutants with low peroxidase activity to achieve near wild-type cyclooxygenase activity once the lag phase is eliminated [24••].

The identity of the 'physiological' hydroperoxide activator is uncertain, but several possibilities exist. Several different fatty acid hydroperoxides react with COX to generate Compound I, so lipid hydroperoxides are likely activators [40,41•]. Peroxynitrite, the coupling product of NO and superoxide anion, is also an efficient substrate for the peroxidase of both COX-1 and COX-2 [42]. It activates the cyclooxygenase activity of either enzyme in the presence of very high concentrations of glutathione peroxidase and glutathione, and activates COX-1 in intact smooth muscle cells [43]. Treatment of lipopolysaccharide-activated macrophages with membrane-permeant superoxide dismutase mimetic agents, which prevents peroxynitrite formation, reduces prostaglandin biosynthesis by up to 85% [42]. An attractive feature of the involvement of peroxynitrite as an activator of cyclooxygenase in inflammatory cells is the fact that both the inducible form of nitric oxide synthase and COX-2 are immediate-early genes that are induced by many of the same agonists and with very similar time courses [44,45]. This provides a regulated pathway for the generation of a hydroperoxide activator coincident with COX-2 expression (Figure 5). In fact, prostaglandin synthesis by activated macrophages from iNOS-knockout mice is significantly reduced compared with synthesis by activated macrophages isolated from wild-type mice [46•].

Enzyme–substrate interactions

Considerable attention has focused recently on the binding of fatty acid substrates in the cyclooxygenase active site. The chemical mandates of the synthesis of a bicyclic peroxide with *trans*-dialkyl substitution require that the fatty acid be bound in an extended conformation with a sharp bend around carbons 10–13 [47]. Modeling this conformation of arachidonate into the cyclooxygenase active site with the carboxylate ion-paired to Arg120 and the 13-*pro(S)* hydrogen adjacent to Tyr385 places the ω-end of the fatty acid in a hydrophobic pocket near the top of the active site (Figure 6; [48••]). A conserved glycine residue (Gly533) is located close to the end of the fatty acid. Mutation of Gly533 to Ala reduces cyclooxygenase activity with arachidonate as substrate by 85% and mutation to Val completely eliminates activity. However, both mutants exhibit undiminished cyclooxygenase activity toward unsaturated fatty acids containing fewer carbons at their ω-end (e.g. α-linolenic acid, stearidonic acid; [48••]).

Confirmation of the importance of the top channel in substrate binding is provided by crystal structures of complexes of arachidonate bound to COX-1 reconstituted with Co^{3+}–heme (W Smith, personal communication) and of PGH_2 bound to apoCOX-2 [11••]. In addition, examination of these structures reveals numerous protein–fatty-acid interactions, suggesting an active role for the protein in controlling the regiochemistry and stereochemistry of arachidonate oxygenation. Of particular

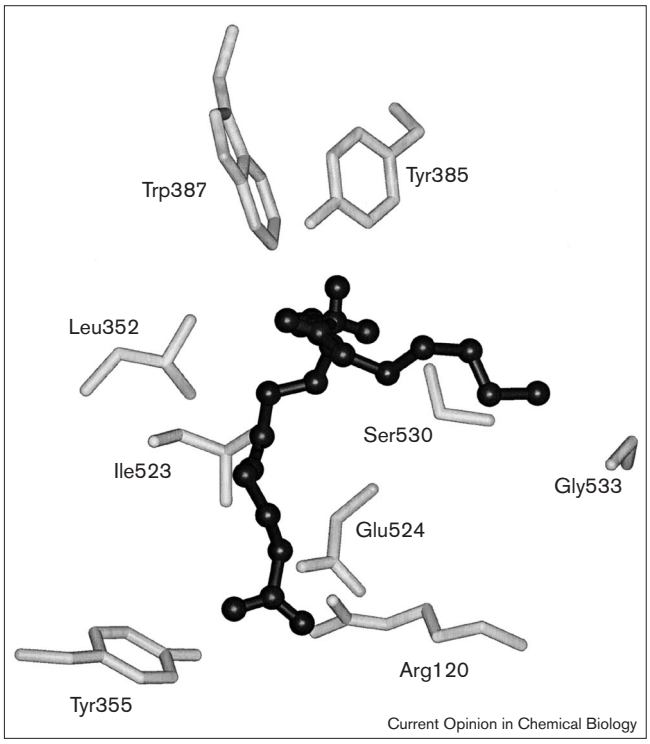
importance is the region around Tyr385 and Trp387. As stated above, the tyrosyl radical derivative of Tyr385 oxidizes the 13-*pro(S)* hydrogen of arachidonate and, as expected, it is positioned close to C-13 in both structures. In addition, Trp387 is close to the endoperoxide group in the COX-2–PGH_2 crystal structure, suggesting that it may restrict the conformation of the 11-peroxyl radical to facilitate cyclization at C-9 [11••]. Indeed, mutation of Trp387 to Phe or Tyr does not abolish oxygenase activity but reduces the yield of PGH_2 20-fold [11••,49]. Crystallography and site-directed mutagenesis also suggest that the protein controls the stereochemistry of O_2 addition to radicals at C-11 and C-15 by steric hindrance ([11••,50]; W Smith, personal communication).

An alternate arachidonate-binding mode is observed in a complex with the His207Ala mutant of COX-2 [11••]. The carboxylate of arachidonate is hydrogen-bonded to Tyr385 and Ser530 and the ω-end projects toward the constriction at Arg120, Tyr355, and Glu524 before bending up toward Leu531. This conformation is inconsistent with catalysis but may correspond to an inhibitory conformation of substrate bound to enzyme.

The cyclooxygenase active sites of COX-1 and COX-2 are very similar but there are subtle structural differences that give rise to functional differences between the two proteins. For example, aspirin acetylation of Ser530 of COX-1 completely inhibits oxygenation of arachidonate by sterically blocking access to the top channel [51]. Aspirin acetylation of the corresponding residue in COX-2 abolishes arachidonate conversion to PGG_2 but not to 15-(*R*)-hydroxyeicosa-5,8,11,13-tetraenoic acid [52]. The greater size of the cyclooxygenase active site in COX-2 apparently allows insertion of arachidonate into the top channel with an altered conformation of both the carboxyl and ω-ends of the molecule; this leads to reversal in the stereochemistry of oxygenation at C-15 [53•,54••].

A more fundamental difference between COX-1 and COX-2 is in the binding of the carboxylate group of the fatty acid substrate. The COX-1–arachidonate and COX-2–PGH_2 crystal structures reveal ionic and hydrogen-bonding interactions with Arg120 and Tyr355, which are located at a constriction point near the bottom of the cyclooxygenase active site and the top of the membrane-binding domains of both proteins ([11••]; W Smith, personal communication). As expected, mutations of Arg120 of COX-1 significantly affect cyclooxygenase activity [55,56]. However, Arg120 mutations in COX-2 are much less deleterious to its cyclooxygenase activity [57,58•]. This suggests that other interactions in the cyclooxygenase active site are more important for binding arachidonate in COX-2 than in COX-1. As a corollary, the carboxylate of arachidonate is not as important for its binding to COX-2 as it is to COX-1. In support of this hypothesis, COX-2 oxygenates the ethanolamide derivative of arachidonic acid (anandamide) to the ethanolamide derivative of PGH_2 [59].

Figure 6



Model of arachidonic acid (black) bound to the active site of COX-1 (gray). Arg120, Tyr355 and Glu524 comprise the constriction that separates the bottom of the COX active site from the lobby in which arachidonate first binds. The constriction must open to permit arachidonate access to the COX active site. Tyr385 sits adjacent to the 13-*pro*(*S*) hydrogen of arachidonic acid and Trp387 facilitates cyclization of the 11-peroxyl radical to form the cyclic peroxide. Ser530 is the aspirin acetylation site and Gly533 is located near the ω-end of the fatty acid.

Conclusions

Recent work from several laboratories has provided important insights into the oxygenation of arachidonic acid by cyclooxygenases. These findings strongly support the chemical mechanism of prostaglandin endoperoxide biosynthesis proposed over 30 years ago by Hamberg and Samuelsson [2] and the biochemical mechanism of cyclooxygenase catalysis proposed 12 years ago by Ruf and co-workers [8]. Reaching this level of understanding has been experimentally challenging because of the short-lived nature of the substrate and enzyme-derived intermediates, the complex interaction between the cyclooxygenase and peroxidase activities, and the unusual kinetics of oxygenation that are complicated by self-catalyzed enzyme inactivation.

We have begun to glimpse views of enzyme–substrate interactions that reveal the identity and role of residues that control regiochemistry and stereochemistry of oxygenation. Furthermore, we are beginning to appreciate the subtle differences in structure between COX-1 and COX-2 that confer distinct substrate specificity and catalytic function. The next few years should witness a

more precise definition of enzyme–fatty-acid interactions for both enzymes. Because COX-2 oxygenates amide derivatives of arachidonic acid, it may be possible to prepare fluorescent substrate analogs that enable real-time monitoring of substrate binding and product release [60]. This should provide a convenient approach for probing the involvement of individual residues in catalysis by both enzymes. Given the roles that COX enzymes play in lipid mediator biosynthesis, it is likely that these structural and functional differences will lead to important physiological consequences.

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- •• of outstanding interest

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Now in press

The work referred to in the text as 'W Smith, personal communication' is now in press:

61. Malkowski MG, Ginell SL, Smith WL, Garavito RM: **Structure of prostaglandin synthase complexed with arachidonic acid.** *Science* 2000, in press.