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Current Topics

The Cyclooxygenase Reaction Mechanism

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The mechanism of the conversion of arachidonic acid (AA)[1] into prostaglandin G_2 (PGG_2) (eq 1) has long fascinated chemists and biochemists alike. In this process, no less than four new chemical bonds are formed, and five chiral centers are introduced into the achiral polyunsaturated fatty acid. Prostaglandin H synthase (PGHS) is the enzyme that accomplishes this impressive feat, which constitutes the first biosynthetic step in the biosynthesis of all prostaglandins as well as of thromboxane and prostacyclin. These bioactive lipids are important modulators of cardiovascular, gastrointestinal, renal, and reproductive function, as well as mediators of inflammation, fever, and allergy, and hence PGHS has been a prime pharmacological target. PGHS is a bifunctional enzyme that first converts AA into prostaglandin G_2 (PGG_2) in the cyclooxygenase (COX) reaction, followed by reduction of the hydroperoxide at position 15 to the corresponding alcohol in the peroxidase reaction (Eq. 1). PGHS is often referred to as COX, after the first reaction it catalyzes. In mammals there are at least two PGHS isozymes (*1*−*4*). These proteins have about 60% sequence identity and are designated PGHS-1 (or COX-1) and PGHS-2 (or COX-2). In a somewhat simplified picture, COX-1 is constitutively expressed and is considered a housekeeping enzyme, whereas COX-2 expression is induced in specific tissues in response to certain stimuli such as cytokines and growth factors (*5*). Over the past five years, important advances have been made in our understanding of the details of the steps involved in the cyclooxygenase reaction, including identification of reaction intermediates and the determinants of the observed stereoselectivity. This review will focus on these recent developments; for comprehensive accounts discussing other aspects of PGHS research, we refer to a number of reviews (*5*−*8*).

CONNECTION BETWEEN CYCLOOXYGENASE AND PEROXIDASE ACTIVITIES

An important question that has been the topic of much debate involves the relationship between the two reactions catalyzed by PGHS. Both activities require heme as cofactor, but many inhibitors of the cyclooxygenase reaction, such as aspirin or indomethacin, do not affect peroxidase catalysis (*5*). On the other hand, PGHS containing Mn protoporphyrin IX instead of heme retains cyclooxygenase activity but displays only very low levels of peroxidase activity (*9*−*12*). Moreover, kinetic investigations demonstrated that cyclooxygenase and peroxidase catalysis occur at distinct sites on the protein (*13*). These observations suggest two independent catalytic processes, but a mechanistic connection between the two activities was recognized early on because cyclooxygenase activity requires hydroperoxide activators (*14*, *15*). Ruf and co-workers (*16*, *17*) provided the first satisfying explanation for these findings. They showed that the interaction of alkylperoxides with the ferric heme in the peroxidase reaction resulted in the formation of a ferryl species and a tyrosyl radial, which was hypothesized to initiate the cyclooxygenase reaction in a distinct active site (Figure 1).

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[1] Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; HPETE, hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; HRP, horseradish peroxidase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGHS, prostaglandin H synthase.

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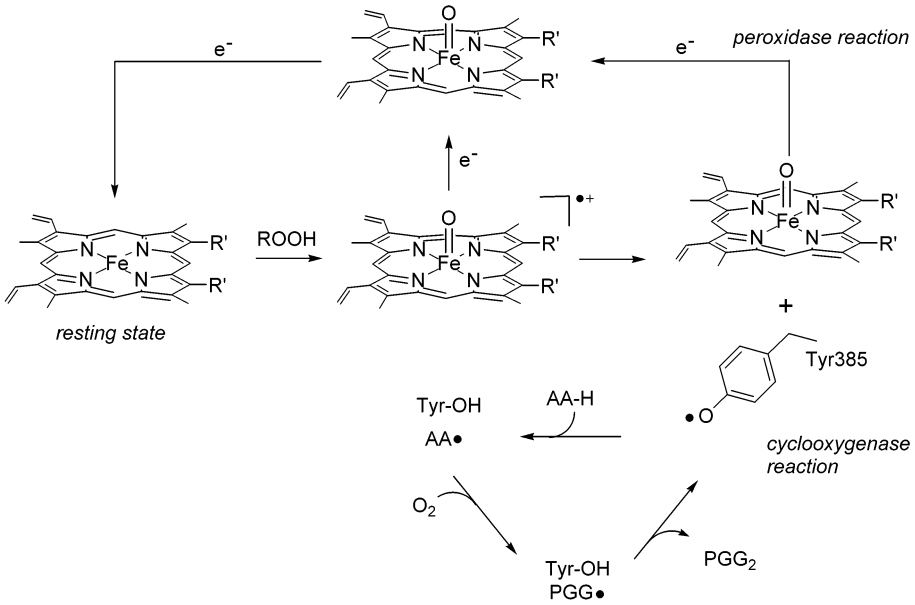
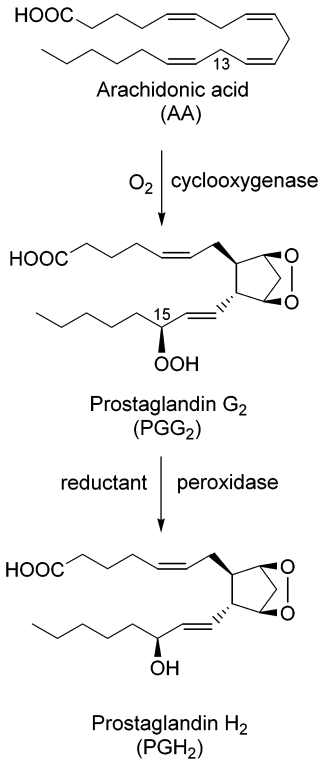


FIGURE 1: The peroxidase reaction generates the tyrosyl radical that is essential for the cyclooxygenase reaction. This process involves two intermediates in which one oxidizing equivalent is stored as a ferryl heme and a second oxidizing equivalent is located on either the porphyrin in Intermediate I or tyrosine 385 in Intermediate II (PGHS-1 numbering). The Fe^{IV}−oxo species is reduced back to the ferric resting state by reducing cosubstrates.

Tyr385 in PGHS-1 was identified as the probable site of the catalytically active tyrosyl radical in studies using chemical and mutagenic modification of the protein (*18*−*20*). The corresponding residue in PGHS-2 is Tyr371. The first crystallographic characterization of PGHS-1 in 1994 confirmed the presence of spatially distinct active sites for the cyclooxygenase and peroxidase reaction (*21*). The peroxidase reaction takes place at the heme, which is situated at the base of a wide, solvent-accessible pocket. The cyclooxygenase active site has been well established through cocrystal



(Eq. 1)

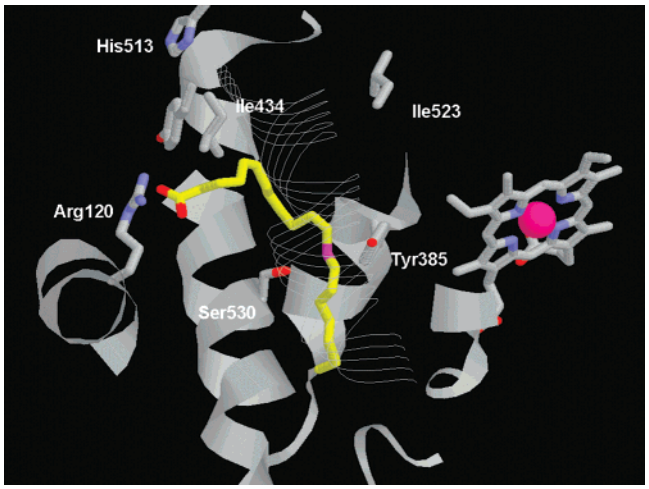


FIGURE 2: Cyclooxygenase active site of Co^{3+}-substituted PGHS-1 with arachidonic acid bound (*22*), PDB accession number 1DIY. Tyr385 is properly positioned to abstract the *pro-S* hydrogen atom from C13 (magenta). Ser530, the site of acetylation by aspirin, is shown, as are the three residues that differ between PGHS-1 and PGHS-2, Ile434, His513, and Ile523. Arg120 is important for substrate binding in PGHS-1 (*74*) but not PGHS-2 (*87*). For clarity, parts of the protein are omitted.

structures of PGHS-1 with inhibitor (*21*) and substrate (*22*) (Figure 2) and of PGHS-2 with product bound (*23*). In accord with Ruf's proposed mechanism, Tyr385 in PGHS-1 (Tyr371 in PGHS-2) is located between the heme and the arachidonic acid binding site, with distances of 12.5 Å between the phenoxyl oxygen and the iron and 2.8 Å between the phenoxyl oxygen and C13 of arachidonic acid. All evidence to date implicates this tyrosine residue as the precursor to the tyrosyl radical that connects the peroxidase reaction with the cyclooxygenase reaction (vide infra).

An important question that was raised by the topology of the two active sites involves the stoichiometric relationship between the two reactions. In Ruf's so-called branched chain radical mechanism, a single reaction of a peroxide with the

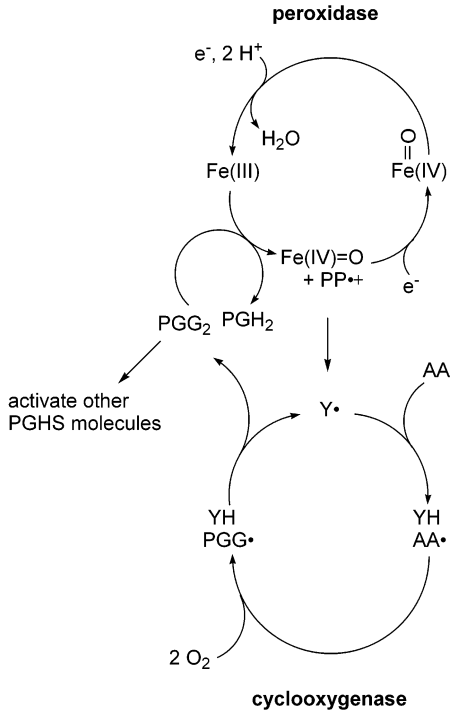


FIGURE 3: Expanded version of Ruf's branched chain mechanism (*26*). Catalysis is initiated by reaction of a hydroperoxide with ferric heme resulting in Intermediate I, which is rapidly converted to Intermediate II containing a ferryl heme and a tyrosyl radical. This radical initiates the cyclooxygenase catalytic cycle and oxidizes arachidonic acid (AA) to PGG_2, which can either be reduced in the peroxidase active site or activate other PGHS enzyme molecules. $PP^{\bullet+}$ = protoporphyrin π cation radical, YH = tyrosine. Not shown is the frequently observed reduction of the tyrosyl radical by reducing cosubstrates, which can dissipate the radical and require a renewed activation step (*36*, *88*).

ferric heme leads to the initiation of multiple turnovers of the cyclooxygenase reaction because the tyrosyl radical is regenerated after each cyclooxygenase catalytic cycle (Figure 3) (*17*). Thus, the PGG_2 hydroperoxide can accumulate and can diffuse away to activate other latent PGHS molecules to their active state, thereby explaining the burst kinetics typically observed in the cyclooxygenase reaction after an initial lag. An alternative mechanistic model, termed the tightly coupled mechanism, requires the reduction of one molecule of hydroperoxide to initiate every turnover of the cyclooxygenase reaction (*24*, *25*). Kinetic studies have supported the branched chain mechanism and demonstrated that once formed, the tyrosyl radical can support multiple cyclooxygenase turnovers independently of peroxidase catalysis (*26*−*28*). Further support for the branched chain mechanism has been obtained with PGHS manipulations that greatly decrease the peroxidase activity but retain near-normal cyclooxygenase activity, including addition of cyanide, replacement of the axial His ligand to the heme by Tyr, and replacement of the heme by Mn protoporphyrin IX (*9*, *29*, *30*). These findings are readily accommodated by the branched chain mechanism but not by the tightly coupled mechanism, in which a reduction in peroxidase activity should diminish cyclooxygenase activity. The cyclooxygenase catalytic cycle does not, however, continue indefinitely because the tyrosyl radical can be dissipated via self-inactivation processes (*31*), by leakage of radical intermediates out of the cyclooxygenase binding pocket prior to regeneration of the tyrosyl radical, or by redox reactions with reducing cosubstrates, thus explaining the ability of peroxide scavengers to suppress cyclooxygenase catalysis even after activation is complete (*15*).

FORMATION OF TYROSYL RADICALS

The peroxide-induced cyclooxygenase activation process has been extensively investigated, and reaction intermediates have been detected and characterized. We will focus here on those aspects of the peroxidase activity that are directly linked to tyrosyl radical generation and cyclooxygenase catalysis. For a more detailed discussion of the peroxidase reaction in mutants and heme substituted PGHS proteins, we refer to recent reviews (*5*, *6*). In its resting state, the PGHS heme is mainly in the ferric high spin state (*32*). Reaction of a hydroperoxide with this ferric heme in PGHS first produces Intermediate I, which has spectral properties very similar to horseradish peroxidase (HRP) Compound I (*17*, *33*). In HRP this intermediate has been shown to consist of an iron(IV)−oxo species and a porphyrin cation radical (*34*). According to the generally accepted mechanism for coupling the events at the PGHS heme to arachidonic acid oxygenation, Intermediate I is converted to Intermediate II by electron transfer from a nearby tyrosine residue (Tyr385 in PGHS-1) to the porphyrin radical cation (Figure 1). The tyrosyl radical so generated can then begin the cyclooxygenase catalytic cycle (vide infra). Such storage of one oxidizing equivalent in the form of a ferryl species and the second oxidizing equivalent on an amino acid side chain is also encountered in cytochrome *c* peroxidase Compound I (also called Compound ES), in which a tryptophan close to the heme is oxidized (*35*). The assignment of Intermediate I in PGHS as the immediate oxidant of the tyrosine is supported by the emergence of the EPR signal associated with the tyrosyl radical concomitant with the increase of the absorption features characteristic of Intermediate II (*36*).

An alternative mechanism has been put forth for PGHS in which the ferric heme oxidizes the hydroperoxide activator to a peroxyl radical, which in turn oxidizes the tyrosine to the tyrosyl radical (*37*). This mechanism was proposed to explain the observation of a spectral intermediate at 446 nm during stopped-flow analysis of the reaction of aspirin-treated PGHS-2 with arachidonic acid. This spectral feature was attributed to the ferrous form of the enzyme, leading to the proposed conversion of the ferric heme to the ferrous heme and concomitant oxidation of the peroxide to the peroxyl radical. Using the ferric heme as an oxidant has some similarities with the proposed oxidation of unsaturated fatty acids by the ferric form of lipoxygenases (*38*). However, extrapolation of this proposal to PGHS raises several issues that have not been adequately addressed to date. First, the Fe(II)/Fe(III) redox potentials in PGHS-1 and -2 have been reported to be −0.052 and −0.156 V, respectively, with only a modest change upon arachidonic acid binding (*29*, *39*). For comparison, this redox potential is significantly higher in soybean lipoxygenase, +0.60 V (*40*). The values for PGHS indicate that the ferric heme is not nearly a sufficiently strong oxidant to oxidize either a hydroperoxide or a tyrosine, as these latter compounds have redox potentials in the range of +0.8−1.0 V (*41*). Intermediate I, on the other hand, appears entirely capable of oxidizing a tyrosine if one takes the reported redox potential of this intermediate in horse-

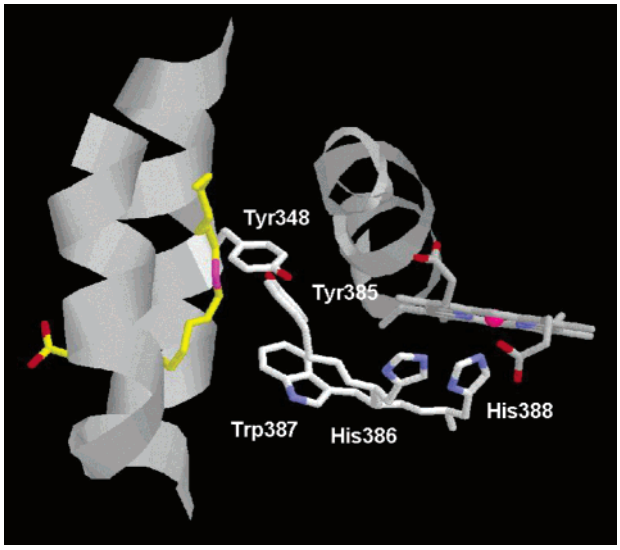


FIGURE 4: Close-up view of the environment of Tyr385 in Co^{3+}-substituted PGHS-1 (*22*). The hydrogen bonding interaction with Tyr348 is shown, as are the residues that connect Tyr385 to His388, the axial ligand to the Co^{3+}-substituted heme. For clarity, the carbonyl oxygens of the backbone amides are omitted and the nitrogens of the amides are colored gray.

radish peroxidase (at pH 6.5, +0.94 V for compound I/II, +0.94 V for compound II/ferric heme (*42*)) as a reasonable estimate for the corresponding species in PGHS. The fate of the phenoxyl proton of Tyr385 during the generation of the tyrosyl radical and the path of electron transfer are currently unclear. Tyr385 is hydrogen bonded to Tyr348 (Figure 4), but no proteinaceous general base is in the vicinity to accept the hydroxyl proton from Tyr385. The pK_a of a protonated tyrosyl radical is on the order of −2 (*43*), and hence one might argue that active deprotonation is not required. However, the redox potential of tyrosines is strongly pH-dependent, and electron transfer without removing the proton is energetically costly, requiring +1.38 V to produce a protonated tyrosyl radical cation in water and 1.8−1.9 V in a low dielectric environment (*44*, *45*). Indeed in most proteins that generate tyrosyl radicals, a general base is hydrogen bonded to the neutral tyrosine and accepts the proton when the tyrosine is converted to its neutral radical form (*8*). In the absence of arachidonic acid, a water molecule in the substrate binding channel may serve as the general base, but when arachidonic acid is bound, no water molecules are present in the hydrophobic vicinity of Tyr385 in the X-ray structure (*22*). Hence, the exact fate of the phenoxyl proton is currently still unclear. Recent high-field EPR studies have shown that once the catalytically active tyrosyl radical is generated in PGHS-1, its phenoxyl oxygen is hydrogen bonded, probably to Tyr348 (*46*). However, mutagenesis of Tyr348 to Phe resulted in a mutant that still displayed 60−83% of the wild-type cyclooxygenase activity and formed the catalytically active tyrosyl radical (*47*), and therefore the hydrogen bond to Tyr348 does not appear to be critical. The second unclear issue in tyrosyl radical formation involves the electron-transfer pathway. One model postulates that the electron moves from Tyr385 along the polypeptide backbone via His386 and Trp387 to His388, the axial ligand to the heme (*5*). Electron transfer might also proceed from Tyr385 down the polypeptide backbone in the other direction to Asn382 and subsequently to the heme. The Asn382 side chain lies ∼3.5 Å from the heme, similar to the distance to Tyr385, and electron-transfer efficiency along a peptide backbone can be affected by side chain structure (*48*). It is thus of interest that an alanine mutation in PGHS-1 at His386 (in the first pathway) decreases cyclooxygenase acceleration (*49*) and that aspartate or histidine substitutions in PGHS-2 at Thr369 (corresponding to residue 383 in the second pathway in PGHS-1) decreases the efficiency of cyclooxygenase activation by peroxide (B. Bambai, and R. Kulmacz, unpublished observations). Another potential path involves direct electron tunneling from Tyr385 to the vinyl edge of the heme, as the distance between Cδ and Cϵ of Tyr385 to the terminal vinyl carbon is just 4.3−4.6 Å in crystallographic structures.

Tyrosyl radicals also can be generated by peroxide in a PGHS-1 mutant with Tyr385 replaced by Phe, which lacks cyclooxygenase activity, and in PGHS-1 and −2 preparations in which the cyclooxygenase activity is blocked by treatment with inhibitors such as indomethacin or nimesulide (*7*). In these cases, the EPR spectra of the peroxide-induced tyrosyl radicals are narrow singlets, quite distinct from those of tyrosyl radicals in native PGHS-1 and -2, indicating that they have a different conformation of the tyrosine ring with respect to the beta carbon. The residues bearing the narrow singlet radicals have not been identified and might well be Tyr385 (Tyr371), except of course when that residue has been mutated. Both PGHS isozymes have a considerable number of tyrosine residues in the general vicinity of the heme, which might be alternative sites for a tyrosyl radical in the Y385F mutant and in self-inactivated protein; so far, only Tyr348 of PGHS-1 has been ruled out by direct examination (*47*).

INTERMEDIATES IN THE CYCLOOXYGENASE REACTION

Hamberg and Samuelsson were among the first to propose a reaction mechanism for the conversion of polyunsaturated fatty acid into PGG (*50*). Using stereospecifically tritium-labeled fatty acid, it was demonstrated that the *pro-S* hydrogen is selectively removed from C13 of the substrate. Although the identity of the oxidant was not known at the time, the authors proposed that a hydrogen atom was removed to generate a delocalized fatty acid radical that can react with oxygen at C-11 (Figure 5). The resulting peroxy radical was proposed to attack C9, resulting in a localized radical at C8, which in turn reacts with the conjugated diene spanning C12−C15. The allyl radical so generated then reacts stereo- and regio-selectively with a second molecule of dioxygen to provide the C15 peroxy radical. The stereoselectivity of this second oxidation is discussed below in more detail. In Ruf's branched chain mechanism, it is the PGHS tyrosyl radical that abstracts the *pro-S* hydrogen atom from C13 of the fatty acid, and in the last step, the C15 peroxy radical reabstracts a hydrogen atom from the tyrosine, thereby preparing the enzyme for additional cyclooxygenase turnovers without requiring another activation event (Figure 5). Thermodynamically, a tyrosyl radical would be a sufficiently strong oxidant to abstract a hydrogen atom from C13 of arachidonic acid, given a bond dissociation energy of a typical phenol O−H bond of 86 kcal/mol compared to 76−83 kcal/mol for bisallylic C−H bonds (*41*, *51*, *52*).

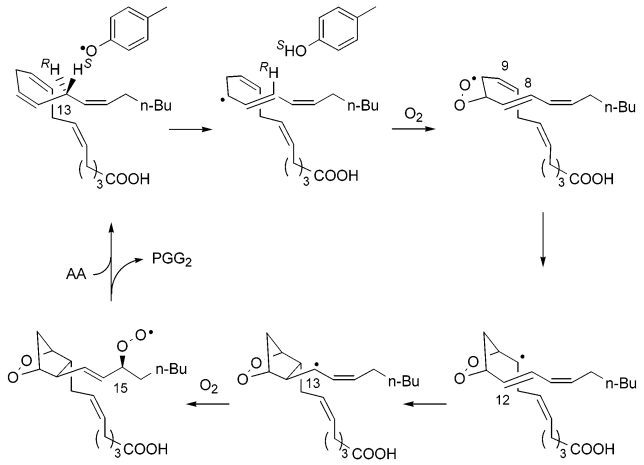


FIGURE 5: Original radical mechanism for the formation of PGG_2 from arachidonic acid (*50*). Hamberg and Samuelsson did not know the origin of the hydrogen abstractor, which was first proposed to be a tyrosyl radical by Ruf and co-workers (*32*).

Early support for generation of substrate radicals during cyclooxygenase catalysis was provided through spin trapping of radicals that diffuse out of the active site of the protein (*53*). More recently, direct support for the first intermediate in Figure 5 has been obtained by EPR spectroscopy (*54*, *55*). Under anaerobic conditions, the tyrosyl radical in PGHS-2 is chemically competent to oxidize arachidonic acid to a substrate-based radical. This radical is located in the cyclooxygenase active site because it has been shown to regenerate the tyrosyl radical upon exposure to oxygen, as predicted by the mechanism in Figure 5. Experiments with synthetic arachidonic acids deuterium labeled at positions 11, 13*S*, 15, and 16 were used to unambiguously identify the intermediate radical species as a C11−C15 pentadienyl radical (*56*, *57*). Furthermore, simulations of the hyperfine values for the individual protons provided the dihedral angles of the respective C−H bonds with the SOMO orbital, thereby defining the conformation of the C9−C17 segment of the intermediate. Although the structure of the radical intermediate formed is well understood in PGHS-2, the situation is less clear for PGHS-1, for which different radical species of currently unknown identity are observed depending on the reaction conditions (*58*). The studies with PGHS-2 provide strong evidence for the generation of a pentadienyl radical under anaerobic conditions, but generation of a C11−C13 allyl radical cannot be ruled out under aerobic conditions. That is, the observation of the pentadienyl radical under conditions in which progress beyond the first intermediate is blocked could be the result of its higher thermodynamic stability, which has been estimated at 11.7 kcal/mol in solution (*59*). Formation of an allyl radical during regular turnover could readily explain the regiochemistry of the first oxygenation at C11 in the generation of prostaglandins. In principle, secondary kinetic isotope effects studies may distinguish between the formation of an allyl or pentadienyl radical during steady-state turnover. Both experimental and theoretical studies have shown a normal secondary isotope effect of 1.06−1.10 in reactions in which an allyl radical is formed from a precursor that is deuterium labeled at the vinyl position (*60*−*62*). Hence, if a pentadienyl radical is formed during steady-state turnover in PGHS, a secondary isotope effect should be observed for 15-[^{2}H]-arachidonic acid.

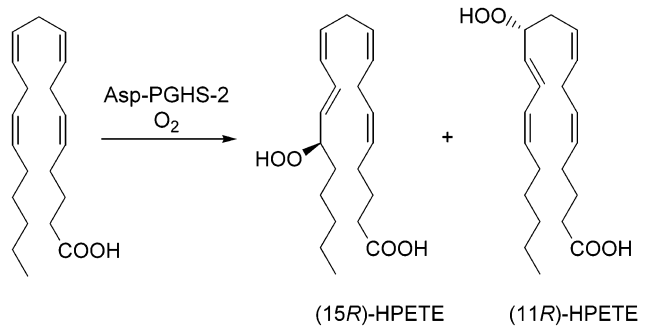


FIGURE 6: Aspirin-treatment of PGHS-2 results in acetylation of Ser516, which in turns leads to an altered product profile. 15-(*R*)-Hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (HPETE) is produced as the major product.

However, if a C11−C13 allyl radical is formed instead, no isotope effect would be induced by a label located at C15. Normal secondary kinetic isotope effects of 1.17 on *V*/*K* have indeed been observed for soybean lipoxygenase using arachidonic acid isotopically labeled at both positions 11 and 15, but the individual contributions of the label at C11 and C15 have not been established (*63*). Similarly, normal secondary kinetic isotope effects have been observed in the reaction of SLO with linoleic acid (*64*). To date no secondary isotope effect measurements have been conducted on PGHS.

STEREOCHEMISTRY OF THE CYCLOOXYGENASE REACTION

In a sense, the chemistry catalyzed by PGHS is simple and "easy". The mechanism in Figure 5 is essentially a series of autoxidations of a polyunsaturated fatty acid that are thermodynamically favorable. Seminal work by Porter and co-workers has shown that the autoxidation of tetraene fatty acids such as arachidonic acid differs from that of more saturated fatty acids in that endoperoxides are formed with tetraenes, as is observed with PGHS, and not with more saturated fatty acids (*65*). Recent theoretical studies on the cyclooxygenase reaction indeed show that once the tyrosyl radical is generated, the cyclooxygenase reaction is an exothermic process (*66*). Hence, the major achievements of the enzyme involve (1) lowering the kinetic barrier imposed by spin conservation rules that normally slows reaction of a spin paired fatty acid with an oxygen diradical, and more importantly, (2) controlling the stereo- and regiochemistry of the oxidations. The factors controlling the selectivity of oxygenation in PGHS have been the topic of much activity in recent years.

Under normal conditions, most prostaglandin H synthases react with arachidonate to produce predominantly PGG_2 with small quantities of (11*R*)-HPETE and (15*R/S*)-HPETE (*67*−*70*). However, a number of reports have documented altered regio- or stereochemical outcomes. Acetylation of Ser516 in the cyclooxygenase active site of PGHS-2 by aspirin changes the product distribution to generate (15*R*)-HPETE and (11*R*)-HPETE as the major products and abolishes prostaglandin formation (Figure 6) (*70*−*72*). On the other hand, acetylation of the homologous Ser530 in the active site of PGHS-1 by aspirin results in a protein that is devoid of any oxygenation activity. Site-directed mutagenesis studies have confirmed the importance of Ser530/516 in governing the stereochemistry at C15. Replacement of this residue with

Met, Thr, or Val in PGHS-2 resulted in mutants that still produced prostaglandins, but with the *R* configuration at C15 (*73*). The stereochemistry at C8, C9, C10, and C11 in the product was unperturbed with these mutants. Furthermore, these mutations enhanced (15*R*)-HPETE formation (*72*, *74*). As found with aspirin treatment, the same mutations rendered the cyclooxygenase activity of human PGHS-1 inactive. Importantly, the stereospecificity of hydrogen atom abstraction from C13 in aspirin-treated PGHS-2 is unchanged compared to that of the wild type (*75*). Recent EPR studies with a variety of deuterated substrates have established that the tyrosyl radical in aspirin-treated PGHS-2 generates a pentadienyl radical, just as in the native enzyme (*58*). Collectively, these studies show that the early events in cyclooxygenase activity in PGHS-2 are unaltered by mutation or acetylation of Ser516, but that the stereoselectivity of oxygenation at C15 is inverted in the prostaglandin product. Brash and co-workers proposed a model to account for this reversal by invoking a 180° rotation along the C13−C14 bond of the substrate induced by the added bulk after acetylation of Ser516 (*75*). This rotation would display the opposite face of the C14−C15 olefin to the incoming oxygen molecule, accounting for the reversed stereochemistry (Figure 7). An altered orientation of the substrate is consistent with the report of different K_m values for the production of PGG_2, (11*R*)-HPETE, and (15*R*/*S*)-HPETE by ovine PGHS-1 (*68*) and human PGHS-2 (*69*). The variation in K_m values suggests that the productive conformations of the substrate leading to each product are distinct. In the Brash model, the substrate radical generated with aspirin-treated PGHS-2 could still be a pentadienyl radical, but with an (*E*,*Z*) conformation instead of an (*E*,*E*) conformation (Figure 7). In solution, these two conformations differ by 2.4 kcal/mol in stability (*59*). As mentioned above, the EPR spectrum of the substrate radical generated in aspirin treated PGHS-2 has been reported (*58*). However, it is difficult to distinguish the (*E*,*E*) and (*E*,*Z*) conformers by EPR spectroscopy because of the small differences in the proton hyperfine values of the isomeric radicals (*76*, *77*) and the large line widths in spectra of frozen solutions. Hence, the proposal of Brash and co-workers awaits experimental verification. An alternative explanation for the reversal of stereochemistry at C15 involves alteration of the direction from which O_2 approaches the radical at C15 (*68*). It is possible that, as in the discussion above regarding the importance of the pentadienyl radical during aerobic turnover, it is actually a C13−C15 allyl radical that forms in aspirin-treated PGHS-2. Such a radical would account for the oxygenation at C15, and either rotation of the ω-portion of the substrate or altered O_2 access could explain the change in stereochemistry. Once more, secondary kinetic isotope effect studies may resolve the issue of whether an allyl radical is indeed generated during steady-state turnover.

The differential behavior of the two isozymes upon treatment with aspirin can be attributed to the ∼20% smaller volume of the fatty acid binding channel in PGHS-1 compared to PGHS-2 (*78*−*80*). This difference is caused by amino acid differences at three positions: Ile434, His513, and Ile523 in PGHS-1 are Val, Arg, and Val, respectively, in PGHS-2. Of these residues, Ile434 lines the substrate-binding pocket, whereas His513 and Ile523 are second-sphere residues (Figure 2). The larger active site in PGHS-2 presumably still permits access of the substrate to the binding

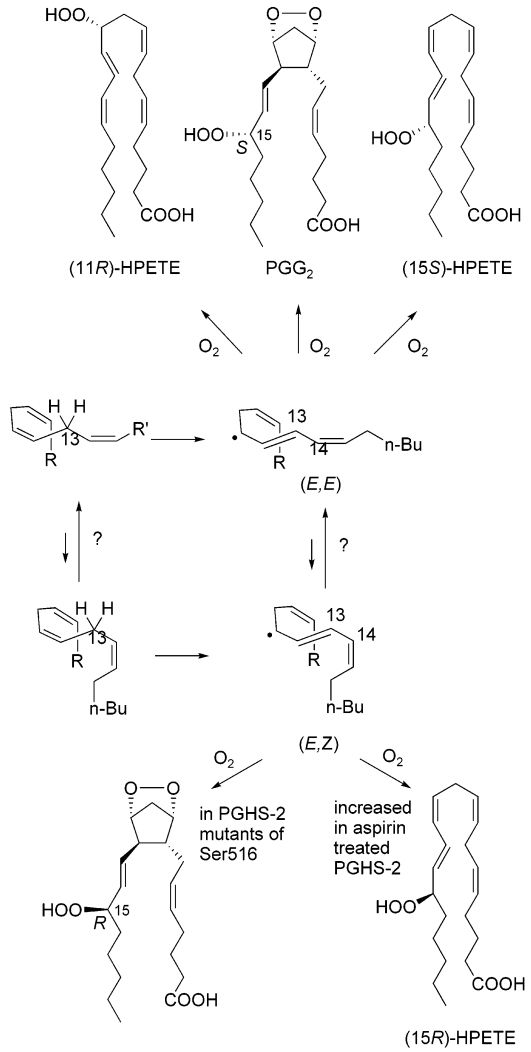


FIGURE 7: Proposed model to account for the variation in stereospecificity at C15. For the production of PGG_2, (11*R*)-HPETE, and (15*S*)-HPETE, the conformation of the C11−C15 fragment is proposed to retain the W-shape seen in the cocrystal structure of Co^{3+}-substituted PGHS-1 and arachidonic acid (Figure 2). For production of 15(*R*)-HPETE (and 15(*R*)-PGG_2) the C11−C15 conformation is proposed to attain a (*E*,*Z*) conformation in aspirin-treated PGHS-2 that exposes the opposite face of the radical at C15 to the putative oxygen binding site. The stereochemistry at C15 in HPETE produced by wild-type human PGHS-2 varies in different studies from predominantly *R* (*69*) to predominantly *S* (*73*). The cause of this variation is unknown but may reflect two interconverting isomeric pentadienyl radicals. The barrier for such interconversion in solution is ∼10 kcal/mol (*59*). Alternatively, two different conformers of the substrate may be bound, consistent with the very different K_m values for PGG_2 and HETE production, 1 and 41 μM, respectively (*69*).

pocket after acetylation of Ser516. Site-directed mutagenesis studies in which all three of these residues in PGHS-2 were mutated to the corresponding amino acids in PGHS-1 resulted in the loss of all oxygenase activities upon aspirin treatment, suggesting that these three residues are indeed responsible for the differential behavior toward aspirin (*81*). Earlier mutagenic experiments in both PGHS-1 and -2 established that the side chain structure of Ile523 in PGHS-1 (Val509 in PGHS-2) is a major determinant of the specificity of COX-2 selective inhibitors (*82*−*84*). A recent comprehensive mutagenesis study, in which 18 residues at or near the cyclooxygenase active site in PGHS-1 were investigated, led

Smith and co-workers to further define the structural factors in the protein that control the cyclooxygenase reaction (*74*). The main conclusions of this effort featured the importance of Tyr348 and Gly533 to position the C13 hydrogen for abstraction by the tyrosyl radical, and the requirement for Val349, Trp387, and Leu534 to direct the substrate radical toward prostaglandin formation as opposed to HPETE production.

SUMMARY AND FUTURE DIRECTIONS

Our understanding of the cyclooxygenase reaction mechanism has vastly improved in the past years through multidisciplinary studies in several laboratories. X-ray crystallography has played a major role in guiding site-directed mutagenesis studies to probe the determinants of product distribution, and the original cyclooxygenase mechanisms proposed by Samuelsson and Hamberg and the branched chain model of Ruf and co-workers have survived recent spectroscopic and kinetic tests. In parallel, impressive advancements have been made in the design of isozyme selective inhibitors that have resulted in highly successful new pharmaceuticals. Several unresolved questions do remain, however, such as the molecular details of self-inactivation, a process that limits PGHS catalysis to several thousand turnovers, and the fate of the phenoxyl proton during tyrosyl radical generation. Furthermore, at present only one of the cyclooxygenase intermediates proposed in the radical mechanism has been characterized, the origin of the selectivity for the initial oxygenation at C11 is still not well understood, and the identity of the peroxide activator of cyclooxygenase catalysis in various physiological settings is the focus of ongoing studies (*85*). Moreover, an intriguing new discovery has recently been reported involving a differentially spliced isozyme derived from the *cox1* gene in dogs (*86*). This protein, designated COX-3, is inactivated by acetaminophen under conditions that do not affect COX-1 and COX-2, possibly explaining the heretofore-unknown mechanism of analgesic activity of this popular drug. COX-3 possesses cyclooxygenase activity, but at present nothing is known yet about its mechanism of catalysis. Clearly, future investigations of PGHS are bound to bring more exciting new discoveries.

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