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PROSTAGLANDIN H SYNTHASE: RESOLVED AND UNRESOLVED MECHANISTIC ISSUES†

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Abstract

The cyclooxygenase and peroxidase activities of prostaglandin H synthase (PGHS)-1 and -2 have complex kinetics, with the cyclooxygenase exhibiting feedback activation by product peroxide and irreversible self-inactivation, and the peroxidase undergoing an independent self-inactivation process. The mechanistic bases for these complex, non-linear steady-state kinetics have been gradually elucidated by a combination of structure/function, spectroscopic and transient kinetic analyses. It is now apparent that most aspects of PGHS-1 and -2 catalysis can be accounted for by a branched chain mechanism involving a classic heme-based peroxidase cycle and a radical-based cyclooxygenase cycle. The two cycles are linked by the Tyr385 radical, which originates from an oxidized peroxidase intermediate and begins the cyclooxygenase cycle by abstracting a hydrogen atom from the fatty acid substrate. Peroxidase cycle intermediates have been well characterized, and peroxidase self-inactivation has been kinetically linked to a damaging side reaction involving the oxyferryl heme oxidant in an intermediate that also contains the Tyr385 radical. The cyclooxygenase cycle intermediates are poorly characterized, with the exception of the Tyr385 radical and the initial arachidonate radical, which has a pentadiene structure involving C11-C15 of the fatty acid. Oxygen isotope effect studies suggest that formation of the arachidonate radical is reversible, a conclusion consistent with electron paramagnetic resonance spectroscopic observations, radical trapping by NO, and thermodynamic calculations, although moderate isotope selectivity was found for the H-abstraction step as well. Reaction with peroxide also produces an alternate radical at Tyr504 that is linked to cyclooxygenase activation efficiency and may serve as a reservoir of oxidizing equivalent. The interconversions among radicals on Tyr385, on Tyr504, and on arachidonate, and their relationships to regulation and inactivation of the cyclooxygenase, are still under active investigation for both PGHS isozymes.

Prostaglandin H synthase (PGHS) is a key enzyme in prostanoid biosynthesis. Mammalian systems have two distinct PGHS isozymes sharing ~60% sequence identity. The constitutive isozyme, PGHS-1, is thought to function usually as a housekeeping enzyme, whereas the inducible isozyme, PGHS-2, is associated with cytokine and mitogen dependent processes, such as inflammation and cell proliferation [1,2]. Both PGHS isozymes catalyze the same two reactions: dioxygenation of arachidonic acid (AA) to yield prostaglandin G₂ (PGG₂), containing both a 9-11 endoperoxide and a 15-peroxide group; and a peroxidase reaction, which converts PGG₂ to prostaglandin H₂ (PGH₂) where the 15-peroxide is reduced to an alcohol

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(Fig. 1) [1]. The product profiles and steady-state kinetics are roughly comparable in the two isozymes, although feedback activation of cyclooxygenase activity by peroxide is considerably more efficient in PGHS-2 [3,4]. The two isozymes are thought to follow the same basic reaction mechanism, so that progress in understanding the PGHS-1 reaction mechanism helps in understanding the PGHS-2 mechanism (and vice versa).

Crystallographic information has been reported for PGHS-1 and -2 complexed with inhibitors and substrates [5-9]. The structure of PGHS-1 containing nonfunctional cobalt protoporphyrin IX and AA is shown in Fig. 2 [6]. A substrate bound structure of PGHS-2 showed that AA also can bind in a nonproductive, reverse-oriented conformation [8]. The core structures of the peroxidase and cyclooxygenase catalytic active sites are very similar in the two isozymes (r.m.s. deviation of 0.4 Å). These crystallographic coordinate sets provide a 3-D framework to guide mechanistic studies. There are two distinct active sites: the peroxidase site identified by heme and the cyclooxygenase site identified by bound substrate or substrate analog. The physical separation of active sites in the crystallographic data fits well with the observation that fatty acids did not inhibit peroxidase catalysis [10,11]. AA binds in a ~25 Å L-shaped hydrophobic channel in PGHS-1, with the fatty acid carboxyl salt-bridged to Arg120 (Fig. 2). It is notable that only three out of the 24 amino acid residues that make up the binding pocket are polar [12]. One of these polar residues, Ser530, is the target of aspirin, and acetylation of Ser530 likely blocks AA binding to PGHS-1 due to steric effects [13]. Heme is the only prosthetic group present, and it is essential for both PGHS activities [14,15]. His388 is the proximal heme ligand and His207 sits ~5 Å from the heme iron on the distal side [5]. This arrangement confirmed earlier conclusions from spectroscopic data that the heme iron is present either in a 6-coordinate low-spin form ligated to a pair of histidines, or in a 6-coordinate high-spin form with a single proximal histidine ligand [16]. Functional PGHS is probably a homodimer anchored on the luminal side of the nuclear and endoplasmic reticulum membranes [1]. Dissociation of the dimer into monomers by treatment with chemical denaturant inactivates both PGHS-1 and -2 [17].

There have been several review articles published about PGHS since 2000 (For example, Refs. [1,18-24]), and many of them touch on the subject of reaction mechanism. This review will focus on describing the process of deciphering the PGHS mechanism, emphasizing controversies that have been resolved and some of the key mechanistic issues that remain unresolved.

PGHS is both self-activating and self-inactivating

PGHS has only one prosthetic group per monomer but the reaction kinetics and mechanism are complex. Cyclooxygenase catalysis requires two substrates, AA and O₂, and peroxidase catalysis takes hydroperoxide and a reductant, or 'cosubstrate' (Fig. 1). Steady-state kinetic assays for cyclooxygenase and peroxidase activities exhibit several unorthodox aspects. Even with excess of all substrates, neither enzyme shows a linear time course (Fig. 3). The peroxidase reaction, monitored by oxidation of a chromogenic cosubstrate, begins at a maximal rate and declines continuously afterward, eventually reaching a plateau without exhausting either substrate or cosubstrate. The cyclooxygenase velocity, monitored by consumption of oxygen, accelerates from zero to a maximal value and then continuously declines back to zero, without exhausting either fatty acid or oxygen. The initial acceleration in the cyclooxygenase reaction reflects autoactivation due to feedback from the product, PGG₂, which is itself a hydroperoxide [22]. The later decay and eventual cessation of both cyclooxygenase and peroxidase reflects the irreversible self-inactivation of each enzyme, thought to originate from oxidative damage to the proteins [18]. The number of catalytic cycles undergone before cyclooxygenase or peroxidase self-inactivation depends on the reaction conditions and can vary from ten to thousands of turnovers [25-27].

Accumulated data from steady-state assays along the lines of those shown in Fig. 3 indicate the PGHS-1 and -2 cyclooxygenases typically have turnover numbers on the order of 10^2 mol O_2 /s/mol monomer and K_M values between 1-15 μM for AA (the wide variation in K_M values is discussed in a later section). The lipid peroxide level required for half-maximal cyclooxygenase activation is ~ 20 nM for PGHS-1 and ~ 2 nM for PGHS-2 [4]. The PGHS peroxidase has a ping pong mechanism [28], and as a result the steady-state V_{max} and K_M values for peroxide vary with the concentration (and structure) of the reducing cosubstrate [29]. With this caveat in mind, the reported PGHS peroxidases turnover numbers range from 10-800 mol PGG_2 /s/mol monomer and the K_M values range from 2-50 μM for lipid peroxide substrates [29-32].

Results from steady-state assays have been useful but they have not been sufficient to address many of the important mechanistic questions. These include how the peroxidase and cyclooxygenase catalytic cycles are coupled, why heme and peroxide are required for cyclooxygenase catalysis, and what happens during self-activation and self-inactivation. Major advances on these issues have come from transient kinetic approaches to monitor redox changes of the heme and protein components, combined with correlated changes in the substrates and products.

Peroxidase reaction mechanism

Rapid-scan stopped flow kinetic experiments and electron paramagnetic resonance (EPR) measurements firmly established that the PGHS peroxidase catalytic cycle is a hybrid of those from horseradish peroxidase and cytochrome c peroxidase [28,33,34] (Fig. 4). PGHS peroxidase catalysis begins with sequential formation of optical intermediates resembling classical peroxidase Compounds I and II. PGHS Intermediate I is a true Compound I, containing $Fe(IV)=O$ and a porphyrin cation radical. PGHS Intermediate II, optically indistinguishable from Compound II, contains $Fe(IV)=O$ and an amino acid radical, similar to the Compound ES found in cytochrome c peroxidase. Dietz et al. [33] found that conversion of Intermediate I to Intermediate II involves intra-molecular electron transfer. Mutagenic studies found that His207 in the distal heme pocket is essential for full peroxidase activity [35,36] (Fig. 5). Poulos and colleagues have proposed a “push and pull” mechanism for peroxidase catalysis [37]. Among the residues surrounding His207 in PGHS-1 is Gln203 (Gln189 in PGHS-2 numbering) that might conceivably serve as the general base and work together with His207 to provide the “pull” to polarize the O-O bond for heterolytic cleavage of the peroxide. For Gln203 to fulfill this role, the side chain needs to have its amide nitrogen H-bonded with His207, a conformation different from that in the crystallographic model [6, 38] (Fig. 5). Recent mutagenic results, however, indicate that removal of the side chain amine group from Gln203 in either PGHS-1 [32] or PGHS-2 [39] has little effect on heterolytic cleavage of peroxide. Thus, His207 may by itself exert sufficient “pull” to achieve peroxide cleavage, so that Gln203 only provide H-bonding to help orient His207. There is no obvious H-bond acceptor on the proximal side of the heme to enhance the electronegativity of the proximal ligand, His388 (i.e., to furnish the “push”). A possible alternative is Tyr504, which is H-bonded to His388 via a structured water molecule [24] (Fig. 5). A push from tyrosine would be much weaker than that from an acidic residue, such as the aspartate or glutamate residue found in the proximal pocket of other peroxidases [37]. A weaker “push” in PGHS is also manifested by the relatively positive heme midpoint potential values reported for the Fe^{+3}/Fe^{+2} redox couple, ranging from -50 to -170 mV [16,31]. These PGHS values contrast with the midpoint potential values for peroxidases that have an acidic residue H-bonded with the proximal histidine ligand, such as cytochrome c peroxidase (-194 mV) [40], horseradish peroxidase (-261 mV) [41], ascorbate peroxidase (-159 mV) [42] and KatG (-222 mV) [43]. In spite of the atypical structure on the proximal and distal heme faces, however, PGHS peroxidase exhibits turnover numbers comparable with other heme peroxidases [44-46].

PGHS peroxidase shows a preference for hydroperoxides with long aliphatic chains, as indicated by the large change of the k_1 value from $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$ for PGG₂, 15(S)-hydroperoxy eicosatetraenoic acid (15-HPETE), *trans*-5-phenyl-4-pentyl-1-hydroperoxide (PPHP) to $\sim 10^6 \text{ M}^{-1}\text{s}^{-1}$ for ethyl hydroperoxide (EtOOH) to $\sim 10^4 \text{ M}^{-1}\text{s}^{-1}$ for H₂O₂ at 1 °C [31-33,47]. Molecular dynamics simulations in PGHS-1 suggest that the alkyl chain (or ring) of a hydrophobic peroxide interacts with residues in the “hydrophobic dome” in the distal pocket, and that the peroxide functional group H-bonds with His207 and Gln203 [32,48]. This “orientation factor” from the hydrophobic interactions, along with the otherwise generally hydrophilic environment of the heme distal pocket, likely promotes ionic (heterolytic) cleavage of the peroxide O-O bond rather than radical (homolytic) cleavage, despite the much larger bond dissociation energy of the former, ~ 360 vs. ~ 40 kcal/mol [32], and the weaker “push-pull” aspect.

Studies of heme replacement with other metalloporphyrins concluded that mangano protoporphyrin IX (MnPPIX) is the only substitution for the PGHS-1 heme that is able to reconstitute at least some catalytic activity, resulting in $\sim 4\%$ of the control peroxidase activity but essentially full cyclooxygenase activity [30,47,49]. Mangano PGHS (Mn-PGHS) thus provides a good model for investigation of the role of peroxidase activity in cyclooxygenase catalysis. When Mn-PGHS-1 is reacted with hydroperoxide, the metal goes through high-oxidation states much like the iron enzyme, and the cyclooxygenase activity is also regulated by the peroxide availability [50,51]. Mn-PGHS-1 has even higher preference than Fe-PGHS-1 for fatty acid peroxides over water soluble peroxides [47,49]. The ratio of k_1 (the rate constant for formation of Intermediate I) for Fe-PGHS-1 compared to the corresponding value for Mn-PGHS-1 is 10^2 for 15-HPETE and 10^4 for EtOOH; the difference has been attributed to slow replacement of the distal water (hydroxide) ligand to Mn(III) PPIX by the peroxide substrate, as found with horseradish peroxidase [52]. However, this proposal is not supported by crystallographic data showing that a glycerol molecule serves as the distal heme ligand in both Fe-PGHS-1 and Mn-PGHS-1; the latter structure does show ~ 0.2 Å more out of plane metal displacement towards the His388 ligand [53]. This difference was suggested to affect the substrate access velocity [53]. Thus, the mechanistic basis for the much weaker peroxidase activity of Mn-PGHS-1 than the iron enzyme remains unsettled.

Sorting out structure and function for the many tyrosyl radicals observed during PGHS catalysis

Early EPR studies found that reaction of pure PGHS-1 with hydroperoxides produced free radical signals, with concomitant decrease of both low-spin and high-spin heme signals (Fig. 6) [34,54]. Karthein et al. [34] noticed that the PGHS-1 EPR signal resembled that reported for the ribonucleotide reductase tyrosine radical [55] (Fig. 6, inset) and proposed that a peroxide-induced tyrosyl radical is the key oxidant in cyclooxygenase catalysis. This “branched chain radical mechanism” for PGHS catalysis has proven to be a very useful model (Fig. 7). In this mechanism, tyrosyl radical generated from the peroxidase reaction serves a central catalytic role, first abstracting a hydrogen atom from the AA substrate and later (as a tyrosyl residue) transferring the hydrogen atom to the PGG₂ radical to form the PGG₂ product. This elegant hypothesis also provided a mechanistic connection between the cyclooxygenase and peroxidase activities, thus explaining why heme is required for both enzyme activities and how peroxide is involved in the feedback self-activation observed in cyclooxygenase catalysis (Fig. 3). As suggested in Fig. 7, an interesting analogy to receptor-mediated signal transduction can be made, considering the peroxide substrate as “ligand”, the PGHS peroxidase as the “receptor”, the tyrosyl radical as the “transducer”, and the cyclooxygenase as the “effector”. Substantial amplification occurs in PGHS across the virtual “boundary” between “receptor” and “effector”, analogous to the transmembrane amplification in receptor-mediated signaling.

Further studies soon led to the realization that four distinct EPR signals could be observed in PGHS-1 reacted with peroxide [21] (Fig. 8). Early in the reaction, the radical signal is a doublet centered at $g=2.0047$ with a 19 G splitting and an overall line width of 34-35 G [56], corresponding to the PGG₂-induced doublet reported by Karthein et al. [34]. This doublet signal (termed “wide doublet” or **WD1**) is transient, becoming a “wide singlet” (**WS1**) with the same peak-to-trough separation [56]. Analogous wide-doublet (**WD2**) and wide-singlet (**WS2**) radicals were found in PGHS-2 reacted with peroxide, but the **WD2** lifetime is much shorter than that of **WD1** and was only detected using rapid-freeze EPR [57].

Pretreatment of PGHS-1 with indomethacin, a stoichiometric cyclooxygenase inhibitor [58] dramatically alters the EPR line shape of the peroxide-derived radical: only a narrow singlet radical signal (**NS1a**) with a 26 G peak-to-trough distance and some subtle hyperfine structure is observed [56]. Another narrow singlet (**NS1b**) is observed in PGHS-1 reacted under highly self-inactivating conditions [59,60]. The EPR line shape of **NS1b** is similar to that of **NS1a** but with less-discernible hyperfine splitting (Fig. 8).

Site-directed mutagenesis and EPR characterization of PGHS-1 and its Y385F mutant, both transiently expressed in COS-1 cells grown in medium containing perdeuterated tyrosine and phenylalanine were conducted to trace the origins of the **WD/WS** and **NS** EPR signals [61]. Deuterium substitution on tyrosine yielded a simplified EPR lineshape for each of the radical species, indicating that they all originated from tyrosyl radicals and not from other species with similar X-band EPR, such as the tryptophanyl radical [61]. This result raised the issue of how a radical on the tyrosine sidechain could produce such a variety of EPR signals. Barry et al. [62] noted that the lineshape difference between **WD1** and **NS1a** can be accounted for by a change in the dihedral angle (θ_1) defined by the p_z orbital of C1 and one of the C-H bonds at the beta carbon of a tyrosyl radical (Fig. 9). In this interpretation, **WD1** has $\theta_1 = 11^\circ$ and **NS1a** has $\theta_1 = 47^\circ$. The physical interpretation of **WS1** was clarified by electron nuclear double resonance (ENDOR) measurements, which indicated that **WS1** is a mixture of **WD1** and a narrow singlet [63], confirming an earlier proposal by DeGray et al. [59]. Careful arithmetic analysis of the EPR data indicates that the narrow singlet radical contributing to **WS1** is likely **NS1b**, found in self-inactivated PGHS-1, and not **NS1a**, found in the indomethacin-treated enzyme [21,63].

Anisotropic g values for **WD1**, **WS1** and **NS1a** were determined by high-field EPR (HFEPR) measurements and the **WD1** was later characterized further by high-field ENDOR (HFENDOR) studies [64,65] (Fig. 10). Resolution of the Zeeman field-related parameter is greatly enhanced in the high-field spectra, whereas the intrinsic nuclear hyperfine splitting remains unchanged. The values of the resolved g_x component, which aligns with the direction of C₁-O bond, strongly indicate that all three radical species are not tryptophanyl radicals and are H-bonded, likely with Tyr348, which is positioned very close to Tyr385 (Fig. 11). The observation of a distribution of g and A values in HFENDOR and HFEPR of PGHS-1 contrasts with the classic tyrosyl radical spectra of ribonucleotide reductase (Fig. 10), and led to the proposal that a progressive Tyr385 phenyl ring rotation is responsible for the **WD1** $\rightarrow$ **WS1** $\rightarrow$ **NS1b** transition in reactions with peroxide [64]. Similar examples of tyrosyl radical conformational changes, namely rotations of tyrosine radical side chains around C _{α} -C _{β} and C _{β} -C₁ (as depicted in Fig. 11), later were found in two Class I ribonucleotide reductases by single-crystal HFEPR [66,67]. In ribonucleotide reductase the rotation about these two bonds was proposed to alter proton-coupled electron transfer between the tyrosyl radical and the di-iron center [66]. If this phenyl ring rotation occurs in PGHS-1, it would be the first example of dynamic conformation changes in a catalytic tyrosyl radical.

An alternative interpretation for the observed EPR lineshape transition in PGHS-1 during reaction with peroxide is formation of radical at another tyrosine residue with a different phenyl

ring conformation. In other words, **NS1b** (and **NS1a**) might originate from a tyrosine residue other than Tyr385. A strategy of mutating possible alternate tyrosyl radical sites near the heme and Tyr385 in PGHS-2 was used to distinguish between the competing hypotheses of Tyr385 radical phenyl ring rotation and alternate radical formation [68]. Electron-transfer pathway analysis was used to identify Tyr148, Tyr404, Tyr348 and Tyr504 as potential alternate radical sites, and thus mutagenic targets (Fig. 11). Four PGHS-2 proteins with single tyrosine → phenylalanine mutations at the target sites, along with one protein with mutations at all four targets (“quadruple mutant”) and another with mutations in all the targets and at Tyr385 (“quintuple mutant”), were overexpressed and purified. Only Y385F and the quintuple mutant completely lost cyclooxygenase activity, while retaining 45-100% of the wildtype peroxidase activity [68]. EPR was used to analyze the peroxide-generated radical signals in each protein. There were no peroxide-induced radical signals in the quintuple mutant (Fig. 12, left panel). In contrast, the Y504F single mutant and the quadruple mutant showed a persistent **WD2** tyrosyl radical, while a **WS2** tyrosyl radical was observed for wild type PGHS-2 and for the single mutants at Tyr148, Tyr348, or Tyr404. A **NS2** was observed for the Y385F single mutant (Fig. 12, left panel). This result strongly supports the “alternate radical” mechanism rather than the “ring rotation” mechanism for PGHS-2 and identifies Tyr504 as the alternate radical site that gives rise to the **NS2** signal. In a recent similar study on human PGHS-1 (hPGHS-1), the Y504F single mutant produced only a **WD** tyrosyl radical upon reaction with peroxide (Fig. 12, right panel) [69]. The Y385F/Y504F double mutant of PGHS-1 had no peroxide-induced radical. These results indicate that tyrosyl radical forms only on Tyr385 or Tyr504 in hPGHS-1, and that Tyr385 is the location of the **WD** tyrosyl radical. However, peroxide reaction with the Y385F single mutant of hPGHS-1 generated only a minute amount of a 10 G singlet and not the expected **NS1** signal [69]. This suggests that a Y385F mutation in hPGHS-1 leads to nonspecific structural changes in this protein, thus leaving the “alternate radical” hypothesis less conclusive for PGHS-1 than for PGHS-2.

Only one peroxide-induced **NS** tyrosyl radical species (**NS2**) has been observed in PGHS-2, whereas ovine PGHS-1 (oPGHS-1) produces three distinct **NS** species, **NS1a**, **NS1b** and **NS1c** (Fig. 8 and Tsai et al. unpublished results). The assignment of the three **NS** species in oPGHS-1 remains ambiguous. One of these presumably results from radical formation at Tyr504, whereas the others may arise from ring rotation of the Tyr385 or formation at Tyr348. There is one established case of Tyr385 radical ring rotation in a PGHS-2 mutant: the peroxide-induced EPR signal from Y348F/Y504F double mutant underwent a time-dependent progression from a **WD2** to **WS2** [70]. This result suggested that removal of H-bonding between Tyr385 and Tyr348 allowed greater conformation flexibility for the Tyr385 radical. The progression from **WD2** to **WS2** in the Y348F/Y504F double mutant is reminiscent of the **WD1** to **WS1** transition observed for oPGHS-1, but it remains to be proven whether tyrosyl radical ring-rotation accounts for any of the **NS** species in oPGHS-1.

Regardless of the remaining uncertainties, there is no evidence so far that peroxide-induced radical intermediates in PGHS-1 and -2 involve any residues other than Tyr385 and Tyr504, even though other oxidizable residues, such as Trp387, are near the heme. Both Tyr385 and Tyr504 are conserved in all known PGHS species, consistent with some evolutionary pressure to retain tyrosines at both positions. The functional role of the Tyr385 radical in specific H-abstraction from AA, as proposed by Ruf and colleagues in their branched-chain radical mechanism (Fig. 7), is convincingly established by direct experimental evidence [18]. The significance of the Tyr504 radical to PGHS catalysis, on the other hand, is just beginning to be evaluated. Y504F mutants in either PGHS-1 or -2 show full cyclooxygenase activity under conditions where peroxide levels are not restricted, but both mutants have impaired cyclooxygenase activity in the presence of glutathione peroxidase-1, a peroxidase scavenger [69]. This observation of decreased cyclooxygenase activation efficiency when formation of Tyr504 radical is blocked suggests that a Tyr504 radical helps to maintain the cyclooxygenase

in its activated state. Crystallographic data [5-9] indicate that a Tyr504 radical would be buried in both PGHS-1 and -2, whereas a Tyr385 radical would be on the surface in the cyclooxygenase pocket, more susceptible to reaction with small reductant molecules. This expected difference in exposure to reductants is consistent with the hypothesis that Tyr504 serves as a stable reservoir of peroxide-derived oxidizing equivalent, decreasing the need for re-activation [69]. Obviously, sorting out the role of the Tyr504 radical and its relationship with the Tyr385 radical will require further evaluation.

The multiple EPR signals of peroxide-induced tyrosyl radicals in PGHS initially led to considerable confusion about the reaction mechanism. Several observations sparked controversies about the functional role of tyrosyl radical. Extensive kinetic studies on both Fe- and Mn-PGHS-1, using EPR analysis of tyrosyl radical intermediates and chemical analysis of product formation [71], produced several observations that appeared to rule out a role for tyrosyl radical in cyclooxygenase catalysis:

- 1) Tyrosyl radical formation lagged behind formation of AA metabolites and instead coincided with cyclooxygenase inactivation.
- 2) Mn-PGHS-1, which had little if any peroxidase activity and very little tyrosyl radical formation, nonetheless exhibited cyclooxygenase activity comparable to that of Fe-PGHS-1.
- 3) Tyrosyl radical levels were substantially attenuated by levels of reducing cosubstrate that did not inhibit cyclooxygenase activity.

These three major observations led to a conclusion that tyrosyl radical is not kinetically competent for cyclooxygenase catalysis and is instead a marker of self-inactivation [71]. This conclusion directly challenged the branched-chain radical mechanism (Fig. 7) and prompted a later modification of the original model to introduce an unspecified radical intermediate, $X^\bullet$, in place of the tyrosyl radical [72].

Resolving this controversy over the functional role of the tyrosyl radical required careful consideration of the underlying basis for the EPR spectral differences among the tyrosyl radical intermediates discussed above. The correlated kinetic measurements for heme redox state, levels of total tyrosyl radical and resolved **WD1/WS1**, total eicosanoid products, and PGG₂ and PGH₂ formation during reaction of PGHS-1 with AA at 0 °C seem to show a correlation of **WD1** kinetics with AA metabolite formation, while **WS1** formation lagged behind (Fig. 13) [60]. Further, rapid-freeze quench EPR kinetic measurements and parallel stopped-flow measurements for heme redox changes (A_{410} for Intermediate I disappearance and A_{580} for Intermediate II formation) in reactions with peroxide demonstrated that oxidized peroxidase intermediates and tyrosyl radicals could be generated very quickly in oPGHS-1, hPGHS-2, and even Mn-PGHS-1 (Fig. 14) [57,73]. These findings offered some reassurance that at least two tyrosyl radicals, **WD1** in PGHS-1 and **WD2** in PGHS-2, were formed from peroxidase reaction intermediates on a time scale consistent with participation in cyclooxygenase catalysis. In addition, it became apparent that the tyrosyl radical species that Lassmann et al. [71] found to be associated kinetically with self-inactivation was not the **WD1** species but most likely **NS1b**, which accumulates under high-turnover conditions and contributes to the **WS1** signal [60]. Thus, issue 1) described above could be resolved satisfactorily in the framework of the branched chain mechanism.

The second issue raised by Lassman et al. [71], that Mn-PGHS-1 has cyclooxygenase activity but not peroxidase activity, also has been resolved by results from subsequent studies. Despite earlier reports that Mn-PGHS-1 was essentially devoid of peroxidase activity [74,75], it was later found that Mn-PGHS-1 actually has a low level of peroxidase activity [50,51,76]. The Mn-PGHS-1 peroxidase activity turns out to be very selective for lipid peroxides and thus is

not readily detected with the HOOH used in the earlier assays [76]. Later, computer simulations of reactions based on numerical integration of mechanistic models were found to predict that the amplification inherent in the branched chain mechanism means that substantial cyclooxygenase catalysis can be sustained even when the peroxidase activity is almost completely inhibited by a heme ligand, cyanide [77]. Finally, several mutants in PGHS-1 and -2 have been found to have very little peroxidase activity yet retain considerable cyclooxygenase activity [31,35,36,78]. These include H204Q in PGHS-1, H386A in PGHS-1 and -2, and H207A and H388Y in PGHS-2. Detailed examination of some of these mutants has revealed that they have slow cyclooxygenase acceleration that can be relieved by added peroxide or compounds that stimulate peroxidase activity. Finally, no PGHS-1 or -2 mutant with cyclooxygenase activity has been established to totally lack peroxidase activity. Overall, the link between cyclooxygenase activation and peroxidase activity predicted by the branched chain model fits well with the available experimental data.

The third argument of Lassmann et al. [71] against a catalytic role for tyrosyl radical was that adding low levels of reducing cosubstrates to reactions of PGHS with peroxide or AA has a stimulatory effect on cyclooxygenase velocity yet greatly attenuates tyrosyl radical levels. A later study found that reducing cosubstrates cause only a modest increase in the actual cyclooxygenase velocity but rather lead to a large increase in the number of cyclooxygenase catalytic cycles before self-inactivation [79]. Elaborations of the branched chain model that allow for independent cycling of peroxidase and cyclooxygenase do predict substantial increases in the cyclooxygenase catalytic lifetime at low cosubstrate levels [79]. These computer simulations also predicted that ~80% of the protein has a tyrosyl radical at the time when peak cyclooxygenase velocity is reached in reactions at cosubstrate levels giving maximal cyclooxygenase stimulation (Bambai and Kulmacz, unpublished).

The simulation results just described suggest that significant levels of tyrosyl radical should be present during cyclooxygenase catalysis, even when cosubstrate is present. This heightens the issue of the susceptibility of PGHS tyrosyl radical to reaction with small molecule reductants. Cells have considerable levels of endogenous cosubstrates for PGHS [25-27] that could quench tyrosyl radical sufficiently to inhibit cyclooxygenase catalysis. Recent observations that some cyclooxygenase substrate analogs greatly stabilize both the tyrosyl radicals in PGHS-2 by decreasing their reactivity with reductants (Kulmacz, Wu and Tsai, unpublished). If similar radical stabilization is found to be triggered by physiological cyclooxygenase ligands it would provide a novel mechanism for stabilization of PGHS tyrosyl radicals for sustaining cyclooxygenase catalysis in spite of abundant cellular reductants.

The cyclooxygenase mechanism of PGHS

The working hypothesis for a free radical-based conversion of AA to PGG₂ during cyclooxygenase catalysis remains essentially the same as that proposed forty years ago by Hamberg and Samuelsson [80] (Fig. 15). The first step is hydrogen abstraction from C13 to form a pentadienyl radical involving C11-C15. In the second stage, O₂ is attacked by the C11 radical, and cyclization forms a 9,11-dioxo bridge and leaves a carbon-centered radical at C8. The third stage involves cyclization between C8 and C12 to generate an allyl radical with significant spin density at C15, which attacks a second O₂ to give a hydroperoxy radical at C15. The final stage is the reduction of the PGG₂ radical to form PGG₂ itself. While this proposal is quite plausible, direct evidence for individual intermediates in the cyclooxygenase cycle itself only started to emerge relatively recently.

a. Identification of the immediate oxidant of AA and structural characterization of the first AA radical intermediate

An anaerobic single-turnover EPR experiment, involving manual addition of a stoichiometric amount of EtOOH to generate the tyrosyl radical before reaction with AA, was used to isolate the first AA-derived carbon-centered radical intermediate [81] (Fig. 15, step 1). The key abstraction of the hydrogen from C13 of AA by the tyrosyl radical was subsequently confirmed using EPR spectrometry. For the experiments with hPGHS-2, the EPR spectra showed sequential formation of the peroxide-derived **WS2** tyrosyl radical then a new broad (~80 G) EPR signal with 7-line hyperfine splitting (Fig. 16, left panel). Thawing of the sample in air to replenish oxygen converted all of them to the **WS2** radical EPR signal (Fig. 16, right panel). The conclusion from these anaerobic single-turnover EPR experiments was that the peroxide-induced tyrosyl radical (**WD** or **WS**) reacts with AA to initiate cyclooxygenase catalysis [81, 82]. In contrast, **NS1a**, **NS1b** or **NS2** generated in inhibitor-treated enzyme or in Y385F mutants (Y371 in PGHS-2 numbering) did not oxidize AA [81,82]. Crystallographic data show that Tyr385 is located between the heme and the AA binding pocket, with the phenoxyl oxygen 2.8 Å from C13 of AA and 12.5 Å from the heme iron (Fig. 2) [6]. However, static crystallographic data have shed little light on the chemical and conformational changes of AA during catalysis. A pentadienyl AA radical structure, with two strongly interacting C10 β-protons and one strongly interacting C16 β-proton (Fig. 17a), was deduced from computer simulation of the single-turnover EPR data and the constraints inherent to formation of the C9-C11 endoperoxide and the 5-membered ring [82]. The formation of a similar pentadienyl AA radical intermediate by Mn-PGHS-1 was confirmed by using d₈-AA to replace AA as substrate [82].

AA radicals have also been trapped inadvertently in cases where oxygen was exhausted during reactions that began under aerobic conditions. A 7-line radical spectrum was observed when concentrated oPGHS-1 (150 μM) was mixed with 25 equivalents of AA [82]. A similar EPR spectrum was observed when 50 μM Mn-PGHS-1 was reacted with 5 mM AA [71]. Formation of the pentadienyl AA radical in both cases can be explained by rapid exhaustion of oxygen due to the high substrate and enzyme concentrations, enabling accumulation of the AA carbon-centered radical [82].

More definitive examination of the structure of the AA radical used a series of specifically deuterated substrates, including 11-d-AA, 13(*R*)-d-AA, 16,16-d₂-AA, 15-d-AA, 13,15-d₂-AA, 10,10-d₂-AA, and 5,6,8,9,11,12,14,15-d₈-AA (Fig. 18) [83]. Replacement of particular hydrogen atoms by deuterium in these substrates generated simplified EPR hyperfine patterns that were consistent with predictions based on the EPR parameter set derived from the original EPR data for unlabeled AA [82] (dashed lines in Figs. 18). These multiple examples of AA radical forming upon reaction of the tyrosyl radical with AA provide direct, convincing evidence for the proposed functional role of the tyrosyl radical in cyclooxygenase catalysis (first step in Fig. 15). EPR data from these experiments with deuterated AA also was used to confirm the conformation of the enzyme-bound AA radical intermediate from C7 to C17 as proposed from earlier results of single-turnover EPR studies [82]. Comparison of this AA radical conformation with those of AA bound to oPGHS-1 [6] and PGG₂ bound to hPGHS-2 [8] (Fig. 17b) suggests that large-scale substrate structure changes occur in the transition from AA to pentadienyl radical to PGG₂. Molecular dynamic (MD) simulations and potential of mean force calculations for AA-13-yl radical binding to oPGHS-1 based on the 1DIY model produced a quite different radical structure from that calculated from the EPR data [84], with notably longer distance between C8 and C12 and C10-C11 torsion (Fig. 17b). If these MD computations can be extended to the millisecond range they may arrive at a conformation closer to that indicated by the EPR characterization. Conversely, MD simulation starting from the

AA radical conformation deduced from EPR data may give some insight into the oxygen access pathway and subsequent transformations.

Specifically deuterated AAs also were used to characterize the substrate-derived radical formed by aspirin-treated hPGHS-2, which has lipoxygenase rather than cyclooxygenase activity [85]. The results indicate that the initial product is likely to be a mixture of a strained allyl radical and a pentadienyl radical. Coincidentally, the EPR signal of this putative allyl radical bears a line shape similar to that of an AA radical found earlier in oPGHS-1 [81], suggesting that there may be a common radical intermediate structure in oPGHS-1 and aspirin-modified hPGHS-2. Further HFEPR characterization of the AA-induced radical intermediate in the aspirin-modified hPGHS-2 needs to be done to verify its origin and structure.

b. AA-induced radical intermediates in PGHS-1

Results from recent extensive anaerobic single-turnover EPR experiments indicate that the production of AA-induced radicals is more complex in oPGHS-1 than previously observed in hPGHS-2, with variable contributions from two additional tyrosyl radical signals, **NS1a** and **NS1c**, in addition to the pentadienyl radical. The **NS1a** is tentatively ascribed to result from AA binding in a non-productive conformation similar to that found for PGHS-2 [8], thus acting as an inhibitor to cause alternate radical accumulation at Tyr504 [86]. The **NS1c** tyrosyl radical signal was previously ascribed to an “allyl radical” [85], but HFEPR spectroscopy (Tsai, A.-L., Wu, G., Rogge, C.E., Lü, J., Peng, S., van der Donk, W.A., Palmer, G., Gerfen, G.J., and Kulmacz, R.J. unpublished) revealed it to be a tyrosyl radical that is distinct from the HFEPR lineshape of **WD1** and **NS1a** [64,65]. The location of this new tyrosyl radical is unclear but could be either Tyr348 or Tyr385 with a relaxed conformation, and probably not Tyr504 as it is too distant for H-transfer.

In a parallel study, formation of AA and tyrosyl radicals during sequential anaerobic reactions of PGHS-1 and -2 with ethyl peroxide and AA was verified by NO trapping experiments, which detected formation of some 3-nitrotyrosine derivatives of the proteins, but larger levels of AA oximes (Lü, J., Rogge, C.E., Wu, G., van der Donk, W.A., and Tsai, A.-L. unpublished). These results indicate that the anaerobic EtOOH/AA reactions produce substantially more AA radical than tyrosyl radical, in contrast with the EPR/HFEPR analyses of similar reactions, which found more **NS1c** tyrosyl radical than AA radical. This apparent discrepancy regarding the proportions of the tyrosyl and AA radicals remains a puzzle. One plausible interpretation is that the **NS1c** and AA radicals equilibrate, with trapping of the AA radical pulling the equilibrium away from the **NS1c** radical, as summarized in Scheme 1.

The redox potential for formation of tyrosyl radical ($\text{Tyr} \rightarrow \text{Tyr radical} + \text{H}^+ + \text{e}^-$) is 0.94 V at pH 7.0 [87]. The corresponding value for formation of a pentadienyl radical ($\text{AA} \rightarrow \text{pentadienyl AA radical} + \text{H}^+ + \text{e}^-$) is only 0.60 V [88]. This large energy difference between the two radicals indicates that direct reversal of PGHS pentadienyl radical formation to regenerate a tyrosyl radical is very unlikely. In contrast, the redox potential for generation of an allyl radical is 0.96 V at pH 7.0 [88]. Thus, conformational changes in PGHS-bound AA radical that led to an allyl AA radical might overcome the energy difference between AA radical and Tyr385 radical. However, reversing AA radical formation still faces a formidable kinetic barrier in the form of large transition state barriers (Fig. 19). However, as discussed in more detail below, observations of significant oxygen kinetic isotope effects [89] are consistent with equilibration of the AA radical and the **WD1** or **NS1c** tyrosyl radicals, as depicted in Scheme 1.

The mechanism in Scheme 1 is a working hypothesis devised to reflect the newer developments and to provide a framework for future investigations. There are several important new elements in this mechanism. The first is the addition of the alternate radical at Tyr504, which was evident

from mutagenic experiments in PGHS-1 and -2 [69,86]. The Tyr385 and Tyr504 radicals can be formed independently during reaction with peroxide, but appear to equilibrate after formation. The proportions of these two tyrosyl radicals differ in PGHS-1 and -2. The alternative tyrosine(s) may serve as temporary storage for the oxidizing equivalent to avoid radical accumulation on the substrate. A second new element is the reversibility of the H-abstraction by the Tyr385 radical to form the AA radical, which was indicated by the observation of oxygen isotope effects [89] and the radical dynamics we observed. The third addition to the PGHS radical picture is the **NS1a/NS1c** tyrosyl radical, which so far has been observed only in anaerobic reactions of PGHS-1 with peroxide and AA. These new tyrosyl radicals are proposed to equilibrate with the Tyr385 radical (WD1), with the equilibrium shifted by the binding of AA (without H-abstraction). The location of the **NS1a/NS1c** tyrosyl radical is not yet known; candidate sites include Tyr504, Tyr348, and Tyr385 (with a different ring conformation from that in the **WD1** radical). The fourth new element in Scheme 1 is the proposed equilibration between the **NS1a/NS1c** tyrosyl radical (with AA bound) and the AA radical. This feature was added to reflect the observation that NO added to PGHS-1 containing the **NS1a/NS1c** radical was able to trap significant amounts of the AA radical as an oxime (Lü, J., Rogge, C.E., Wu, G., van der Donk, W.A., and Tsai, A.-L. unpublished). It is assumed, though not yet shown, that oxygen carries out the same sort of trapping as seen with NO. The final new elements in Scheme 1 are the cyclooxygenase inactivation steps proposed to originate from the Tyr385 radical and the AA radical (shown as dotted lines). Both of these radicals are strong oxidants located in the cyclooxygenase pocket, and thus each could plausibly cause local oxidative damage to the protein resulting in loss of cyclooxygenase activity. Tyr504 radicals are located some distance from the cyclooxygenase pocket and are tentatively excluded from cyclooxygenase inactivation processes. The relative contribution of the two cyclooxygenase inactivation steps is not yet known but may have important implications. As noted above, **NS1a/NS1c** appears to be the major radical species in PGHS-1 under anaerobic conditions, whereas the AA radical predominates in PGHS-2 under similar conditions. As a consequence, cyclooxygenase inactivation via the AA radical would have a disproportionate effect on PGHS-2, under conditions where oxygen levels are limiting. This may be relevant to tissues with compromised circulation.

There are many unresolved aspects of the interconversions in Scheme 1 to investigate. These aspects include the balance of the individual equilibrium steps, the relative contributions of the AA radical and the individual tyrosyl radicals to cyclooxygenase inactivation, and the impact that the interconversions between radicals have on the physiological functions and regulation of the two isoforms. Answering these questions will be an important part of understanding how PGHS-1 and -2 control and direct the powerful oxidants generated during cyclooxygenase catalysis.

c. Identification of other radical intermediates in cyclooxygenase catalysis

The pentadienyl AA radical is the first substrate-based radical to be verified experimentally in the proposed cyclooxygenase mechanism (Fig. 15), and so far it is also the only such intermediate to be identified. Trapping AA radicals for spectroscopic characterization can be done relatively simply by removing oxygen, whereas trapping later radical intermediates will require development of additional strategies. One approach may be to add substoichiometric amounts of oxygen after generation of the AA radical to trap the 11-peroxyl radical or subsequent radical intermediates, such as the C8 or C15 carbon centered radicals (intermediates X and Y in Fig. 15). Another general approach is to use synthetic AA analogs modified to block cyclization to the endoperoxide or formation of the cyclopentane ring. Specifically fluorinated AA derivatives offer some promise in this regard [90]. Similarly, a 7-thia AA analog might trap the C8 carbon-centered radical [91], based on the logic of resonance delocalization

by an adjacent π -system to stabilize the radical, as was applied to the mechanistic study of lysine 2,3-aminomutase [92].

Another general approach to trapping cyclooxygenase radical intermediates is to tune the protein active site structure by site-specific mutagenesis in order to stabilize a particular intermediate. Extensive structure/function studies have been conducted using site-directed mutagenesis of the primary and secondary shell residues of the AA binding channel [12, 93-95]. Many of these mutations decrease formation of the endoperoxide ring and/or the C8-C12 cyclopentane ring, producing 11(*R*)- and 15(*S*)-H(P)ETE, sometimes with inverted chirality at C11 or C15 [93,94]. PGHS-2 with modifications at Leu384 or Gly526 formed several very unusual products, including 8,9,11,12-diepoxyalcohols, in addition to 11(*R*)-HETE [93]. Three 8,9,11,12-diepoxyalcohol products accounting for more than 50% of the total yield were found in the reaction catalyzed by few of the Gly526 mutants [93]. Formation of the diepoxyalcohols suggests arrest at the C8 carbon-centered radical (Intermediate X in Fig. 15), before C8-C12 bond formation. Thus, PGHS-2 mutants at Gly526 or Leu384 may prove useful in mechanistic studies.

d. Oxygen binding in cyclooxygenase catalysis

The cyclooxygenase mechanism in Fig. 15 involves activation of the fatty acid substrate rather than activation of molecular oxygen. In fact, oxygen enters the picture only after formation of the reactive AA radical at C11. This implies that oxygen collides with the radical and promptly reacts to form a peroxy radical, without formation of a ternary complex between AA, oxygen and enzyme. This scenario has been questioned based on PGHS-1 steady state oxygen electrode measurements, which indicated that the $V_{\max}/K_M$ value for O_2 varied as a saturable function of fatty acid level, and the $V_{\max}/K_M$ value for fatty acid also varied as a saturable function of the O_2 level [89]. This behavior was interpreted to support a random sequential mechanism in which oxygen and fatty acid bind to PGHS-1 before H-abstraction occurs. However, the $V_{\max}/K_M$ value for O_2 also varied as a saturable function of the level of phenol, behavior that is not expected from the mechanism. Reducing cosubstrates such as phenol have been shown to have large stimulatory actions on the number of cyclooxygenase catalytic turnovers before self-inactivation but only modest effects on the cyclooxygenase velocity, once corrections were made for oxygen electrode response characteristics [79]. Mukherjee et al. used uncorrected oxygen electrode rate values [89]. Thus, it is unclear what portion of the responses of the $V_{\max}/K_M$ values to oxygen and fatty acid levels was actually due to changes in self-inactivation kinetics, and whether correction for oxygen electrode effects would alter the mechanistic conclusions.

e. Rate-limiting step(s) of cyclooxygenase catalysis

Identifying the rate-limiting step(s) in cyclooxygenase catalysis is a fundamental part of understanding the chemical mechanism. Once activated, cyclooxygenase cycles independently of peroxidase catalysis and so the limiting step in cyclooxygenase catalysis is not in the peroxidase cycle. There is evidence indicate that the initial H-abstraction (step 1 in Fig. 15) can be rate-limiting. Hamberg and Samuelsson detected a tritium isotope effect by analysis of the $^3H/^{14}C$ ratio in unreacted fatty acid after incubation of microsomal oPGHS-1 with 13 pro-*S* - 3H , 3- ^{14}C 8, 11, 14-eicosatrienoic acid [80]. After 64% conversion the 3H enrichment in unreacted substrate was 226%, and at 75% conversion the enrichment was 284%. The value of the isotope effect derived from these enrichment values was calculated by later workers [89] as 3.1 for 64% conversion and 4.2 for 75% conversion, indicating that removal of the 13 pro-*S* hydrogen of AA was rate limiting. Recent measurements of oPGHS-1 and hPGHS-2 cyclooxygenase velocities with unlabeled AA and 13 pro (*S*)-, 13- d_2 -, or 10,10,13,13- d_4 AA indicated a primary kinetic isotope effect (KIE) (Dk_{cat}) of 1.7-2.4 (Wu, G., van der Donk, W.A., Kulmacz, R.J. and Tsai, A.-L. unpublished results). This range of KIE values for deuterated

AA is consistent with the tritium KIE of ~4 calculated from the earlier results [80,89]. However, the deuterium and tritium KIE values for PGHS are much smaller than the intrinsic value, ~7 for H/D and ~15 for H/T, which could indicate either a significant suppression by the forward and reverse commitment or that one or more other steps is at least partially rate-limiting [89].

Two-tier quantum mechanics calculations of the activation free energy landscape of cyclooxygenase intermediates (Fig. 19) [96] predicted that there are only two steps with elevated activation $\Delta G^\ddagger$: 14.5 kcal/mol for the H-abstraction step and 14.9 kcal/mol for cyclopentane ring formation. This identifies these steps as potentially rate-limiting for cyclooxygenase catalysis. Furthermore, the activation energy barrier for reversing the H-abstraction step is predicted to be 23.7 kcal/mol (Fig. 19), the largest of any step, suggesting that the H-abstraction step is irreversible.

The conclusion that the first H-abstraction step is the only rate-limiting step has been challenged by the recent observation of an ^{18}O KIE of 1.013-1.014 in cyclooxygenase catalysis with AA or linoleic acid [89]. These oxygen KIE values are comparable to the predicted equilibrium isotope effect for formation of a peroxy radical intermediate and a hydroperoxide product, consistent with a peroxy radical being formed in or before the first kinetically irreversible step [89]. This implies that the microscopic chemical steps before reaction with oxygen, including the H-abstraction of AA by tyrosyl radical, are reversible. The authors suggested that either the endoperoxide ring formation or the C8-C12 crosslinking (steps 3 and 5, respectively, in Fig. 15) could be rate-limiting. The recent observations on the AA-induced radical dynamics, summarized in Scheme 1, are consistent with reversible H-abstraction. Moreover, the substrate D/H or T/H KIE or the oxygen $^{16}\text{O}/^{18}\text{O}$ KIE only reflect the events from the free substrate to the first irreversible step and are not sensitive to the subsequent steps down to the end product release. With the small KIE values determined, it is very likely that there are multiple steps with comparably slow rates limiting the overall catalytic process.

The earlier observation of a tritium isotope effect indicating that H-abstraction is rate limiting [80] appears to be at odds with the more recent observation of an oxygen isotope effect indicating that steps before reaction with oxygen are reversible [89] and further work is needed to resolve the disparity. Nevertheless, the relatively modest tritium isotope effect observed for the cyclooxygenase already sets the H-abstraction process in PGHS-1 apart from H-abstraction in two other fatty acid oxygenases, where much larger isotope effects were observed. One of these is the fatty acid alpha-oxygenase from rice, which like PGHS-1 and -2 is a member of the myeloperoxidase family [97] and is thought to use a tyrosyl radical in catalysis [98]. The rice alpha-oxygenase showed an extraordinarily large deuterium KIE of ~50, which was attributed to a hydrogen tunneling mechanism, clearly pointing to H-abstraction as the first irreversible step [99]. The second example of a fatty acid oxygenase exhibiting a large KIE is soybean lipoxygenase (LOX), which has non-heme iron as the catalytic oxidant [100]. In the case of LOX, primary deuterium KIEs as large as 30-50 have been observed in both non-competitive and competitive experiments [101,102]. Here again, the large KIE points to H-abstraction as the rate-limiting step and a hydrogen tunneling mechanism has been generally accepted [103]. The much smaller primary KIE for H-abstraction in PGHS indicates the process of generating the initial fatty acid radical is controlled by a very different mechanism from that in rice alpha-oxygenase and in LOX, and that hydrogen tunneling may be not involved in the H-abstraction step of PGHS-1 although the tunneling mechanism cannot be ruled out. Instead, the KIE of PGHS-1 seems most likely to be due to zero point energy difference between hydrogen and deuterium.

Cooperative aspects of PGHS catalysis

The homodimeric structure of both PGHS isozymes introduces the possibility of allosteric interactions between the monomers. The conventional approach to testing for allosteric effects is to look for cooperative responses of enzyme activity to changes in substrate level. Many groups have examined the AA and oxygen dependence of cyclooxygenase activity of purified PGHS-1 and -2 using steady-state measurements (Table 1). Simple saturable behavior has been consistently reported for O₂ and for AA at substrate levels above 1-2 μ M. Cyclooxygenase K_M values for oxygen (in reactions with AA) range from 5 to 13 μ M for the two isozymes (Table 1). Oxygen is thus present in excess in typical in vitro assays with air-saturated buffer, but might become limiting in tissues if the circulation is compromised. Cyclooxygenase K_M values for AA obtained with purified enzyme preparations range from 1.9 to 15 μ M for PGHS-1 and 0.9-14.7 μ M for PGHS-2 (Table 1). Some of this substantial variation in reported K_M values may reflect differences in assay conditions.

Swinney et al. [104] were the first to perform a detailed analysis of cyclooxygenase activity extending below micromolar levels of AA. Their surprising result was that oPGHS-1 exhibited cooperative changes in cyclooxygenase activity in response to the substrate level, whereas hPGHS-2 showed simple saturable cyclooxygenase kinetics. This cooperativity in PGHS-1 was attributed to an allosteric effect of AA binding, but a detailed mechanism was not proposed. However, later studies found that heme replacement by MnPPIX increased PGHS-1 cyclooxygenase cooperativity and changed PGHS-2 cyclooxygenase kinetics from saturable to cooperative, and that peroxide addition decreased or abolished cyclooxygenase cooperativity in PGHS-1, Mn-PGHS-1, and Mn-PGHS-2 [105]. Further, kinetic simulations predicted that cyclooxygenase cooperativity depends on the hydroperoxide activator requirement and initial peroxide concentration, consistent with observed behavior [105]. It thus appears that the PGHS-1 cyclooxygenase cooperativity at low AA levels [104] originates in the feedback activation kinetics of the branched chain radical mechanism (Fig. 7) and that the cooperativity difference between the isozymes can be explained by the difference in feedback activation loop efficiency.

In vivo, both PGHS isozymes are likely to encounter a complex mixture of fatty acids with divergent structures and activities as cyclooxygenase substrates. Of particular interest are mixtures of n-6 highly unsaturated fatty acids (HUFA), which tend to be efficient substrates in solo reactions, and n-3 HUFA, which by themselves tend to be non-substrates in the absence of added peroxides [106,107]. The results from a recent study comparing the effects of mixed fatty acids on oPGHS-1 and hPGHS-2 suggested that addition of a non-substrate fatty acid in combination with AA tends to inhibit oPGHS-1 catalysis, but to enhance hPGHS-2 activity [108]. It was hypothesized that the binding of some non-substrate fatty acids to the first, “allosteric” monomer of PGHS-2 increases the cyclooxygenase activity once AA binds to the other, “catalytic” monomer. The stimulatory effect on PGHS-2 activity is modest, with a maximum increase of ~60% for the fatty acid combinations tested, and its physiological significance remains to be investigated [108]. It might be noted that the cooperativity linked to fatty acid is observed with PGHS-2, in contrast with the peroxide-linked cooperativity, which is observed only in PGHS-1 [104].

Substrate channeling

Feedback activation of latent cyclooxygenase, with Tyr385 in resting state, to active cyclooxygenase, with a Tyr385 radical, requires movement of product PGG₂ peroxide from the cyclooxygenase site to the peroxidase site on the same subunit or the other subunit within a PGHS dimer, or on a separate PGHS dimer. The Tyr385 radical can be quenched by endogenous or exogenous reductant, and such deactivation is an ongoing process and requires

reactivation with additional peroxide, as demonstrated by the ability of glutathione peroxidase to quench catalysis even after full activation [109]. PGG₂ moving from cyclooxygenase site to peroxidase site within a dimer (termed “channeling”) would have an increased effective local concentration and be less susceptible to interception by external peroxidases, and would thus lower the peroxide level needed to keep the cyclooxygenase activated. Routine cyclooxygenase assays operate under $V_{\max}$ conditions and are not sensitive to channeling. However, isotope dilution experiments by Eling's group found PGG₂ channeling in microsomal PGHS-1, but not in the detergent solubilized enzyme [110], indicating that the channeling required the protein to be in its native membrane environment. In this connection, crystallographic studies have noted voids in the PGHS-2 dimer with the potential to carry PGG₂ [8]. Electrostatic potential calculations also identified a channel with abundant positively charged residues that might facilitate the PGG₂ channeling from the cyclooxygenase site to the peroxidase site [111]. Given the potential effect of channeling on activation, it might be worthwhile to further examine PGG₂ channeling and cyclooxygenase activation in both PGHS isozymes in both membrane-bound [112] and solubilized states.

Half-of-sites reactivity

Titration of oPGHS-1 with heme and competitive cyclooxygenase inhibitors indicated that occupancy of just one monomer in the oPGHS-1 dimer was sufficient to exert maximal effect [58,113]. However, this half-of-sites behavior was not supported by subsequent crystallographic data showing fatty acid or substrate analog bound to both monomers in the PGHS-1 and -2 dimers [5-9]. Similarly, radiation inactivation results indicated that the minimal functioning unit of PGHS-1 is the monomer [114]. Thus it was surprising when a PGHS-2 heterodimer comprising one wildtype monomer and one monomer with a G533A mutation known to block cyclooxygenase catalysis displayed the same cyclooxygenase specific activity as the wildtype homodimer [115]. In the same study, a PGHS-2 heterodimer comprising one wild type monomer and one monomer with a R120Q mutation that blocks cyclooxygenase inhibitor binding displayed comparable cyclooxygenase inhibition as seen for wild type homodimer. There is thus considerable direct evidence for half-of-sites behavior in both PGHS-1 and -2, though the structural basis for the phenomenon has yet to be identified. It will also be important to determine whether the half-of-sites aspect is just an interesting biochemical oddity or has some functional significance.

The heterodimer approach also has been used to test whether peroxide-derived oxidant in one monomer can form the Tyr385 radical for cyclooxygenase catalysis by the other monomer [32]. This experiment combined a hPGHS-2 monomer with a G533A mutation, which blocks cyclooxygenase activity [108], and a monomer with a Q203R mutation, which blocks both peroxidase and cyclooxygenase activities (the cyclooxygenase presumably being lost only because peroxidase catalysis is absent)[35]. The resulting G533A/Q203R heterodimer retained substantial peroxidase activity but showed no cyclooxygenase activity even though one monomer had no mutation in the cyclooxygenase site. This suggests that oxidized peroxidase intermediates in one monomer are not able to generate tyrosyl radical in the other monomer, though it is still possible that the Q203R mutation has an undetected effect on cyclooxygenase site structure. Transient kinetic characterization of such heterodimer PGHS to look into the redox changes of the heme and tyrosyl radical and correlated with substrate and product changes should give decisive answer to this issue of half-of-site activity.

There are indications that PGHS-1 and PGHS-2 monomers can associate to form active enzyme *in vivo* [116], though the oligomeric state remains to be assessed directly, and the physiological significance has not been explored.

Self-inactivation of PGHS

a. Dissecting peroxidase and cyclooxygenase inactivation processes

The feedback activation aspect of the branched chain radical mechanism gives PGHS-1 and -2 the potential to generate large amounts of potent signaling molecules. Thus, stringent regulatory mechanisms are needed to prevent deleterious effects. The ultimate limit to production of prostanoids is the irreversible self-inactivation of both enzyme activities [25], and this limit is evident from the time courses of the reactions (Fig. 3). For the purified isozymes, the number of turnovers before inactivation varies between 10 to 1300 depending on the concentration of reductant and other factors [25,27]. As indicated in the peroxidase and cyclooxygenase mechanisms (Figs. 4 and 15), there are several radical and high-oxidation state heme intermediates that could plausibly lead to oxidative damage and irreversible inactivation. Evaluating the actual role of each such intermediate requires methods of separately monitoring the inactivation kinetics for cyclooxygenase and for peroxidase. Direct analysis of the oxygen consumption kinetics (Fig. 3, right panel) provides one way to monitor cyclooxygenase inactivation. However, decreases in cyclooxygenase activity can be due to reversible decreases in Tyr385 radical level as well as to irreversible damage at either the peroxidase or the cyclooxygenase site. Developing specific assays for cyclooxygenase site damage has turned out to be nontrivial and remains an interesting challenge. On the other hand, the nature of the PGHS mechanism makes it relatively simple to isolate peroxidase inactivation kinetics from those of the cyclooxygenase. PGHS peroxidase catalysis does normally involve exogenous reductant, or cosubstrate (Fig. 4). To simplify the issue, one approach has been to first characterize peroxidase self-inactivation in the absence of cosubstrate. PGHS does have a limited amount of endogenous cosubstrate, which permits multiple turnovers [117] and thus substantially simplifies data interpretation. This approach avoids the many complications in interpreting steady-state data for oxygen consumption to analyze peroxidase inactivation, or using cosubstrate consumption to monitor cyclooxygenase inactivation [118-120].

As noted above, depletion of substrate or cosubstrate can contribute significantly to the decay of peroxidase velocity. This complication can be minimized by a two-stage experimental design, in which inactivation occurring during a first stage is assessed in a second stage assay with excess reactants. Manual implementation of the two-stage approach provided an early examination of peroxide-driven inactivation kinetics [27]. However, the inactivation process is relatively rapid, making the manual mixing time a significant factor, and the manipulations involved make extensive kinetic analyses very time consuming. The sequential stopped-flow instrument (Fig. 20) has proven useful for overcoming these limitations [121]. In this approach, PGHS is first mixed with the hydroperoxide being tested, aged for various lengths of time (first stage reaction), and then mixed with excess H_2O_2 and guaiacol to assay the surviving peroxidase activity (second stage reaction). One example of the use of this method is shown in Fig. 21, where $0.5 \mu\text{M}$ oPGHS-1 was reacted with $250 \mu\text{M}$ EtOOH in the first stage. Increasing the duration of exposure of PGHS to peroxide in the first stage ("aging time") increases the inactivation of peroxide, as is evident from the progressively smaller bursts of peroxidase activity observed in the second stage time courses (Fig. 21A). The initial slope of a given time course provides the surviving peroxidase activity for the corresponding aging time. A secondary plot of surviving peroxidase activity vs. aging time produces a decay curve (Fig. 21B), allowing calculation of the first order rate constant for the particular first stage conditions.

The sequential stopped-flow approach accommodates wide variation in the identity and concentration of peroxide in the first stage. Further, the kinetics of peroxide-driven cyclooxygenase can be analyzed using 11,14-eicosadienoic acid instead of H_2O_2 /guaiacol in the second stage and monitoring oxygenation of the fatty acid by the increase in A_{235} [122].

b. Identification of intermediate II as the starting point of peroxidase inactivation

The peroxidase inactivation kinetics of oPGHS-1 have been analyzed over a range of peroxide and enzyme concentrations for both water-soluble peroxides (H_2O_2 and EtOOH) and lipid-soluble peroxides (15-HPETE and PPHP) (Fig. 22). In each case, inactivation followed a first order process, with a rate of $0.2\text{--}0.5\text{ s}^{-1}$ that appeared to be largely independent of the structure and concentration of peroxide, and the enzyme level (Fig. 22) [121]. This behavior indicates that the peroxidase self-inactivation process originates after the peroxide-dependent formation of Compound I and probably with Intermediate II, which contains an oxyferryl heme and a tyrosyl radical. This finding contrasts with results from early single-stage experiments where the inactivation rate depended on the peroxide concentration [29]. From precedents with other heme peroxidases [123,124], a dependence on peroxide level suggests an inactivation process originating with peroxidase Compound I or Compound III. However, such a process does not seem plausible with PGHS because the equivalent of Compound I, Intermediate I, transforms very quickly into Intermediate II (most estimates of k_2 in Fig. 4 are $>10^2\text{ s}^{-1}$ [32,47,125], and a PGHS analog of Compound III has not been observed.

c

Rapid scan experiments were used to analyze the electronic absorbance changes during peroxidase self-inactivation [121]. The results identified two new, spectrally distinct species, termed “Intermediate III” and “Terminal Compound”, and indicating that peroxidase inactivation follows a linear, two-step mechanism in which Intermediate II is first converted at $0.5\text{--}2\text{ s}^{-1}$ to Intermediate III, which undergoes a slower ($0.01\text{--}0.05\text{ s}^{-1}$) transition to the terminal species (Fig. 23). Chromatographic analysis of Intermediate III indicated that it retains an intact heme group that is not covalently linked to the PGHS-1 protein, though peroxidase activity was not restored by addition of exogenous heme [121]. It thus appears that Intermediate III has suffered damage to the protein in the peroxidase site.

The peroxidase inactivation mechanism appears to be largely unchanged by binding of cyclooxygenase inhibitor, such as indomethacin or flurbiprofen, or heme substitution with MnPIX [126]. The rate of peroxidase inactivation was about 0.3 s^{-1} in inhibitor-treated PGHS-1 and much slower in MnPGHS-1 (0.05 s^{-1}), but the rate remained independent of peroxide and enzyme concentrations, consistent with the mechanism in Fig. 23. One interesting difference was that significant chemical modification of the heme to iron chlorine or metalloporphyrin-protein adducts were found for the inhibitor complexes of PGHS-1 and MnPGHS-1 [126].

d. Peroxide-driven inactivation of cyclooxygenase activity

The sequential stopped-flow approach was applied to analysis of cyclooxygenase inactivation during incubation of oPGHS-1 with peroxide [122]. In the absence of added reducing cosubstrates, which maximizes the levels of oxidized enzyme intermediates expected to lead to inactivation, cyclooxygenase activity was lost as fast as, or somewhat faster than, peroxidase activity, with rates of $0.2\text{--}1.0\text{ s}^{-1}$. In contrast with the peroxidase results, cyclooxygenase inactivation kinetics appeared to be sensitive to the structure of the peroxide used. Interestingly, including a reducing cosubstrate (guaiacol) during reaction of oPGHS-1 with peroxide protected the peroxidase activity to a much greater extent than the cyclooxygenase activity (Fig. 24) [122]. In the absence of guaiacol, the two activities exhibited parallel, biphasic inactivation. Including guaiacol blocked almost all of the peroxidase inactivation but provided only partial protection for the cyclooxygenase. The results reveal that peroxide-derived oxidants can produce distinct types of damage at the peroxidase and cyclooxygenase active sites. Identifying the nature of this damage, and its relationship to oxidants at the heme and at Tyr385, remains an ongoing challenge.

e. Identification of oxidant involved in peroxidase inactivation

As mentioned above, Intermediate II was shown to be the immediate precursor for peroxidase self-inactivation. Intermediate II contains two strong oxidant species, oxyferryl heme and tyrosyl radical, and it is of some interest to understand the roles of each of these two oxidants individually. Covalent linkage formation between amino acid radical intermediate and the heme leading to enzyme inactivation has been documented for ascorbate peroxidase and KatG, a multifunction catalase/peroxidase [127-129]. To address this, the kinetics of tyrosyl radical, oxyferryl heme, and peroxidase inactivation were examined in reactions of various hydroperoxides with oPGHS-1 reconstituted with heme or MnPIX (Fig. 25) [73]. Tyrosyl radical formation was significantly faster with 15-HPETE than with EtOOH and roughly paralleled oxyferryl heme formation at low peroxide levels. However, the oxyferryl heme decayed much more rapidly than the tyrosyl radical at higher peroxide levels (20 equivalents). Inactivation of the peroxidase activity during the reaction of oPGHS-1 with EtOOH or 15-HPETE correlated with oxyferryl heme decay, but not with changes in tyrosyl radical intensity or EPR line shape, indicating that the oxyferryl heme, and not the tyrosyl radical, is probably responsible for peroxidase inactivation in these reactions (Fig. 25). Computer modeling to a minimal mechanism indicated that the oxyferryl heme could account for peroxidase inactivation without contribution from tyrosyl radical [73]. The lack of correlation between tyrosyl radical intensity and peroxidase inactivation was also observed for the **NS1a** tyrosyl radical formed in the indomethacin-treated oPGHS-1 [73]. These results have led to a working hypothesis that the culprit for peroxidase inactivation is the high-oxidation state heme, whereas the **WD1/WS1** tyrosyl radical may be either directly or indirectly involved in damage at the cyclooxygenase site. The observation that peroxidase inactivation kinetics were not altered by a Y385F mutation in oPGHS-1 [118] fits with this hypothesis.

While a basic pathway of peroxide-driven inactivation has now been outlined for PGHS-1 (Fig. 23), the information covers only a small portion of the PGHS inactivation picture. One major gap in understanding regards inactivation during reaction of PGHS with fatty acid substrates, where effective tools need to be developed to distinguish peroxidase site events from cyclooxygenase site events. Another important unresolved area is the mechanism underlying the sensitivity of inactivation kinetics to fatty acid structure, such as the large differences in inactivation with n-3 and n-6 substrates [130,131]. There is also the issue of possible differences in inactivation processes between the two PGHS isozymes. Perhaps most fundamental is the need to identify the peroxidase and cyclooxygenase site residues in PGHS-1 and -2 that are damaged during inactivation.

SUMMARY—In the last decade, consensus was reached that a branched chain radical mechanism involving a catalytic tyrosyl radical is an appropriate mechanistic model to explain the nonlinear reaction kinetics of the cyclooxygenase and peroxidase activities of PGHS-1 and -2, and the coupling between the two activities. There have been new insights into the complicated dynamics of peroxide- and AA-induced radical intermediates, and into these radicals' origins and their possible functions in cyclooxygenase activation and peroxidase self-inactivation. Progress has been made in identifying the reversible and rate-limiting steps in cyclooxygenase catalysis. However, little is known about the details of the cyclooxygenase catalytic cycle and cyclooxygenase inactivation. There are also differences between PGHS-1 and -2 in the interconversions of radical intermediates that remain to be explained in mechanistic terms and related to the catalytic behaviors and physiological roles of the two isozymes. These issues should provide a stimulating challenge for researchers over the next decade.

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ABBREVIATIONS

PGHS	prostaglandin H synthase
oPGHS-1	ovine PGHS-1
hPGHS-2	human PGHS-2
LOX	lipoxygenase
WD	wide doublet tyrosyl radical
WS	wide singlet tyrosyl radical
NS	narrow singlet tyrosyl radical
EPR	electron paramagnetic resonance
HFEP	high-field EPR
ENDOR	electron nuclear double resonance spectroscopy
HFENDOR	high-field ENDOR
AA	arachidonic acid
HUFA	highly unsaturated fatty acid
PGG ₂	prostaglandin G ₂
PGH ₂	prostaglandin H ₂
TMPD	<i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine
KIE	kinetic isotope effect
MnPPIX	mangano protoporphyrin IX
EtOOH	ethyl hydroperoxide
15-HPETE	15(S)-hydroperoxy eicosatetraenoic acid
PPHP	<i>trans</i> -5-phenyl-4-pentenyl-1-hydroperoxide

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Scheme 1.

A hypothetical model summarizing the interconversions of the various tyrosyl radical species and the AA radical in PGHS-1 and -2. The EPR signatures are indicated for each of the tyrosyl radicals, along with the assigned location of the radical; the AA radical (AA•) is the pentadienyl species with a 7-line EPR signal. Parallel reactions for formation of the Tyr385 and Tyr504 radicals from Intermediate I are indicated by the yellow arrows at the top. AA reacts with the Tyr385 radical to form the AA radical (AA•); in PGHS-1, AA can also bind to the Tyr385 radical (without H-abstraction) to form the **NS1a/NS1c** radical, thought to be located on Tyr504, Tyr348, or Tyr385 (conformation differs from that in **WD1**). O₂ reacts with the AA• to eventually form PGG₂. In trapping experiments, NO reacts with the AA• to form oximes. The specificity of individual steps to one isozyme or the other is indicated by color coding. Cyclooxygenase inactivation steps are shown as dashed red arrows.

Figure 1. Reaction of PGHS with AA showing the cyclooxygenase and peroxidase steps. Changes in the atoms derived from molecular oxygen are highlighted with dashed lines.

Figure 2.

Structure of AA bound to PGHS-1 from crystallographic data (1DIY) [6]. Heme was replaced by cobalt protoporphyrin IX (shown in green); AA (backbone shown in cyan) interacts electrostatically with Arg120, with C13 of the fatty acid adjacent to Tyr385; and Ser530, the target of aspirin, is nearby. The locations of the membrane anchor helices and the trypsin digestion site, Arg277, are also indicated.

Figure 3.

Time courses for peroxidase (left) and cyclooxygenase reactions of PGHS-1. The peroxidase reaction with HOOH was monitored by A_{436} increases due to oxidation of the reducing cosubstrate, guaiacol. The cyclooxygenase reaction with AA was followed by measuring oxygen uptake with a polarographic electrode [143]. The peak velocity in each reaction is indicated by a dashed line.

Figure 4.

Current PGHS peroxidase mechanism. The generally accepted mechanism [33] is a hybrid of the mechanisms of horseradish peroxidase and cytochrome c peroxidase. The formal oxidation state of the heme iron is indicated. ROOH and ROH, hydroperoxide and corresponding alcohol; PPIX and PPIX^{•+}, protoporphyrin IX and its cation radical; e⁻, reducing equivalent from peroxidase cosubstrate; Y[•], tyrosyl radical.

Figure 5.

Structural model of key residues in PGHS peroxidase catalysis, based on crystallographic data for PGHS-1 containing MnPPIX (2AYL) [53]. The protoporphyrin is shown in yellow, with Mn as an orange sphere. The structured water H-bonded between Tyr504 and His388 is shown as a cyan sphere. Distances are indicated in Å.

Figure 6.

X-band EPR spectra of oPGHS-1 before (red) and after (blue) reaction with EtOOH. The resting enzyme has prominent signals from high-spin ferric heme (HS) and low-spin ferric heme (LS), along with a small signal from non-specific ferric iron (NI). The heme signals are decreased in the peroxide-reacted PGHS-1, replaced by the tyrosyl radical signal (RAD) with a g value of ~ 2 . Inset: expanded view of radical signal showing the hyperfine features. The resting PGHS-1 spectrum is essentially featureless in the $g = 2$ region when recorded at the low modulation amplitude used for peroxide-treated PGHS-1. Reproduced with permission from Ref. [21] (Copyright 2000 Elsevier Science Inc.).

Figure 7.

The “branched-chain” reaction mechanism linking PGHS peroxidase and cyclooxygenase. The model originally proposed by Ruf and colleagues [33,34] is adapted here to indicate that the PGHS peroxidase (POX) might be considered a “receptor” for peroxide “ligand”, leading to formation of the “transducer”, the Tyr385 radical, and thus activating the “effector”, cyclooxygenase catalysis. An imaginary boundary between “receptor” and “effector” is indicated by a dashed line.

Figure 8. X-band EPR signals of peroxide-induced tyrosyl radicals observed in oPGHS-1 and hPGHS-2. Assignments for individual signals are shown in the table at right.

Figure 9.

Geometry of the radical tyrosyl side chain. Left: illustration of the localization of the unpaired electron at C1, C3 and C5, and showing the key dihedral angle, θ , and the direction of the three principal g components. Right: numbering of carbon atoms.

Figure 10.

Comparison of HFEPR and x-band EPR spectra of **WD1**, **WS1**, and **NS1a** radical signals from oPGHS-1 with spectra of the tyrosyl radical in ribonucleotide reductase. HFEPR spectra were recorded at 190 and 285 GHz, and x-band spectra (insets) at 9 GHz. The **WD1** spectra are superimposed on the ribonucleotide reductase Tyr• spectra in the lower right panel. Reproduced with permission from Ref. [64] (Copyright 2000 American Chemical Society).

Figure 11.

Components of PGHS tyrosyl radical dynamics. Structure of oPGHS-1 (1DIY; [6]) illustrating the position of Tyr385, which is H-bonded to Tyr348, Tyr504, Tyr148, Tyr404, the potential alternate tyrosyl radical site, and bound AA, which has C13 shown in green. Potential conformational changes in the Tyr385 sidechain are indicated by arrows. Heme is replaced by CoPPIX in this structure.

Figure 12.

EPR spectra of hPGHS-1 and -2 mutants supporting alternate radical formation at Tyr504. Samples of hPGHS-2 constructs (A) and hPGHS-1 constructs (B) [68] were reacted with EtOOH and manually freeze trapped before the EPR spectra were recorded [68,69]. Reproduced with permission from Ref. [69] (Copyright 2009 Elsevier Science Inc.) and from Ref. [68] (Copyright 2004 American Chemical Society).

Figure 13.

Evidence for kinetic competence of **WD1** tyrosyl radical in oPGHS-1. Correlation of oxidized heme intermediates (decrease in A₄₁₀ and increase in A₅₈₀), **WD1** (doublet) tyrosyl radical, and cyclooxygenase product (PGG₂ + PGH₂, total eicosanoids) during aerobic reaction of oPGHS-1 with AA [60]. Reproduced with permission from Ref. [60] (Copyright 1992 American Society for Biochemistry and Molecular Biology).

Figure 14.

Evidence for kinetic competence of **WD1** tyrosyl radical in oPGHS-1. Top panel: Rapid freeze quench EPR kinetic measurements showing rapid formation of **WD1** in reactions of oPGHS-1 with EtOOH [57] and the time-dependent spectral transition to **WS1**. Bottom: Kinetics of tyrosyl radical concentration (spin/heme) and ferryl heme levels (A_{524}). Reproduced with permission from Ref. [57] (Copyright 1999 American Society for Biochemistry and Molecular Biology).

Figure 15.

Hypothetical mechanism for cyclooxygenase catalysis, modified from the original proposal by Hamberg and Samuelson [80] to include the role of the Tyr385 radical ($Y^\bullet$) in generation of the first AA radical intermediate ($AA^\bullet$) and to reflect the possible reversibility of the initial steps (blue arrows). Anaerobic conditions block at step 2.

Figure 16.

EPR spectra supporting generation of the first AA radical intermediate by PGHS-2 tyrosyl radical (step 1 in Fig. 15). PGHS-2 was mixed sequentially with EtOOH and then AA under anaerobic conditions before freeze-quenching and EPR analysis [82]. The spectrum obtained before addition of AA (a) shows the **WS2** signal reflecting a mixture of Tyr385 and Tyr504 radicals [68]. The spectra obtained at shorter and longer times after addition of AA (b, c) show the 7-line hyperfine splitting pattern consistent with an AA pentadienyl radical [82]. Thawing and adding oxygen to these samples regenerated the **WS2** tyrosyl radical EPR (b' and c'). Radical intensities are given in spin/heme. Reproduced with permission from Ref. [82] (Copyright 1998 American Society for Biochemistry and Molecular Biology).

Figure 17.

Structure of the initial AA radical in cyclooxygenase catalysis. Panel **a**: Conformation of C8-C16 of the pentadienyl AA radical deduced from EPR characterization and additional constraints from the geometry of the C9-C11 endoperoxide and the C8-C12 crosslink [82]. The locations of the six strongly-interacting protons are highlighted, along with the spin distribution and the key dihedral angles at C10 and C16. Panel **b**: Comparison of the AA radical conformation deduced from EPR analysis (center) with the protein-bound structures of AA (left, 1DIY) and PGG₂ (right, 1DDX). C1-C7 and C17-C20 of the AA radical were arranged manually to approximate their positions in the AA and PGG₂ structures.

Figure 18.

EPR spectra of AA radicals produced by hPGHS-2. Peroxide-treated hPGHS-2 was reacted anaerobically with unlabeled AA or with the indicated deuterium-substituted AA. Spectral simulations using the EPR parameter set in Ref. [82], adjusted for deuterium substitution, are shown in dashed lines. Pink dots in the structures indicate the deuterated sites, and the hyperfine splitting patterns are indicated at right.

Figure 19.

Calculated free energy landscape of the cyclooxygenase catalysis in PGHS based on hybrid density functional theory (AM1/UB3LYP) [96]. Potential rate-limiting steps are indicated by red arrows and reverse steps with high activation barriers are indicated by green arrows. Reproduced with permission from Ref. [96] (Copyright 2003 American Chemical Society).

Figure 20.

Sequential stopped-flow device used to analyze PGHS peroxidase and cyclooxygenase inactivation kinetics. The “substrate” syringe for the second stage reaction holds excess H_2O_2 +guaiacol (to assay surviving peroxidase activity) or excess 11,14-eicosadienoic acid (to assay surviving cyclooxygenase activity).

Figure 21.

Effect of peroxide concentration on oPGHS-1 peroxidase self-inactivation kinetics. Sequential stopped-flow experiments with 7.8-250 μM EtOOH in the first stage used the device shown in Fig. 20. **Panel A:** Second-stage time courses of surviving peroxidase activity (monitored by oxidation of guaiacol at 436 nm) are shown for the indicated durations of oPGHS-1 first-stage incubation with 250 μM EtOOH. The surviving peroxidase activity was quantified from the maximal slope of each time course. **Panel B:** Surviving peroxidase activities measured in the second-stage reaction are plotted as a function of the first-stage aging time with each of the indicated levels of EtOOH. First order rate constants were estimated by fitting the data to an exponential equation. Reproduced with permission from Ref. [121] (Copyright 1999 American Society for Biochemistry and Molecular Biology).

Figure 22.

Effects of peroxide structure and oPGHS-1 concentration on peroxidase self-inactivation kinetics. The first order rate constants determined for peroxidase self inactivation are shown as a function of peroxide concentration for HOOH (A), EtOOH (B), PPHP (C) and 15-HPETE (D). Inactivation kinetics were measured at several levels of oPGHS-1 (enzyme concentrations are indicated alongside the symbols) for each peroxide. Adapted with permission from Ref. [121] (Copyright 1999 American Society for Biochemistry and Molecular Biology).. Symbols refer to measured rates at different enzyme concentrations.

Figure 24.

Cosubstrate effects on peroxide-induced inactivation of peroxidase and cyclooxygenase activities in oPGHS-1. Sequential stopped-flow experiments were used to monitor the decay of peroxidase and cyclooxygenase activities during incubation with peroxide ($60 \mu\text{M}$ EtOOH) in the presence or absence of cosubstrate (0.50 mM guaiacol). A one-phase fit was used for decay with guaiacol present and a two-phase fit was used for decay without guaiacol. Reproduced with permission from Ref. [122] (Copyright 2003 American Chemical Society).

Figure 25.

Comparison of decay kinetics for tyrosyl radical (Tyr•), oxyferryl heme (A₄₂₈) and peroxidase activity (POX) during reaction of 30 μ M oPGHS-1 with 20 equivalents of either EtOOH (top; Ref.[73]) or 15-HPETE (bottom). Reproduced with permission from Ref. [73] (Copyright 2007 American Chemical Society).

Table 1Cyclooxygenase K_M values for arachidonate and oxygen in purified mammalian PGHS-1 and -2.

Protein	Substrate	K_M (μ M)	Method	Reference
oPGHS-1	AA	1.9 $\pm$ 0.2	Direct, O ₂	[89]
oPGHS-1	AA	2.7 $\pm$ 0.2	Coupled, HVA ¹	[132]
oPGHS-1	AA	3.0 $\pm$ 0.9	Direct, O ₂	[106]
oPGHS-1	AA	3.7	Direct, O ₂	[105]
hPGHS-1	AA	4.5 $\pm$ 1.6	Direct, O ₂	[133]
hPGHS-1	AA	10.2 $\pm$ 1.5	Direct, AA	[104]
oPGHS-1	AA	11	Coupled, HVA	[134]
oPGHS-1	AA	15	Direct, O ₂	[135]
oPGHS-1	AA	15	Direct, O ₂	[136]
hPGHS-2	AA	0.9 $\pm$ 0.1	Coupled, HVA	[132]
hPGHS-2	AA	1.1	Direct, O ₂	[137]
hPGHS-2	AA	1.7 $\pm$ 0.4	Direct, O ₂	[106]
hPGHS-2	AA	2	Direct, O ₂	[105]
hPGHS-2	AA	5	Direct, O ₂	[108]
hPGHS-2	AA	5.1 $\pm$ 2.2	Direct, O ₂	[133]
hPGHS-2	AA	9	Direct, O ₂	[138]
hPGHS-2	AA	9.2	Direct, O ₂	[108]
hPGHS-2	AA	10.0 $\pm$ 1.1	Direct, AA	[104]
hPGHS-2	AA	14.7	Coupled, TMPD	[139]
oPGHS-1	O ₂	5	Direct, O ₂	[140]
oPGHS-1	O ₂	5.5 $\pm$ 0.8	Direct, O ₂	[89]
oPGHS-1	O ₂	10 $\pm$ 1.2	Direct, AA	[141]
oPGHS-1	O ₂	11-12	Direct, O ₂	[142]
mPGHS-2	O ₂	13 $\pm$ 1.4	Direct, AA	[141]

¹ HVA: homo-vanilic acid