Issues in Biochemistry

Tyrosyl Radicals and Their Role in Hydroperoxide-Dependent Activation and Inactivation of Prostaglandin Endoperoxide Synthase†

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Received September 19, 1991; Revised Manuscript Received November 7, 1991

Prostaglandin endoperoxide (PGH) synthase catalyzes the formation of prostaglandin endoperoxide H_2 (PGH_2) from arachidonic acid [Figure 1; see Smith et al. (1991) for a recent review]. This is the central reaction in prostaglandin biosynthesis. In intact cells, arachidonic acid, cleaved in a hormone-dependent manner from glycerophospholipids, is converted to PGH_2 through the action of PGH synthase, and then, depending on the cell type, PGH_2 is enzymically isomerized or reduced to PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxane A_2 (TxA_2), or prostacyclin (PGI_2). PGH synthase is important therapeutically because it is selectively inhibited by aspirin and related nonsteroidal antiinflammatory drugs (Vane & Botting, 1987), and its formation may be modulated at the transcriptional and/or translational levels by antiinflammatory steroids [reviewed by DeWitt (1991)].

Formation of PGH_2 from arachidonate involves two distinct reactions, both of which are catalyzed by PGH synthase [see Smith and Marnett (1991) for a recent review]—a bis-oxygenase reaction forming PGG_2 and a peroxidase reaction involving the reduction of PGG_2 to PGH_2 (Figure 2; Miyamoto et al., 1976; van der Ouderaa et al., 1977; Pagels et al., 1983). PGH synthase 1[1] has a subunit molecular weight of 72 000, and it appears to function as a homodimer. The enzyme is an integral membrane protein of the endoplasmic reticulum. It contains three asparagine-linked, high-mannose carbohydrate groups (van der Ouderaa et al., 1977; Mutsaers et al., 1985).

Importantly, there appears to be one heme group per subunit of active PGH synthase 1, and titration of the apoenzyme with heme leads to coactivation of both the cyclooxygenase and peroxidase functions. Thus, a single heme is both necessary and sufficient for the functioning of both enzyme activities [see Smith and Marnett (1991) for a review]. The heme appears to be liganded at the axial position by the imidazole group of His309[2] (Figure 3; Shimokawa & Smith, 1991); there is also speculation that His388 is the distal heme ligand and that His207 may interact via an ionic linkage with a heme carboxyl side chain.

Resolution of Cyclooxygenase and Peroxidase Activities. Despite their codependence on heme, it is possible to resolve the cyclooxygenase and peroxidase activities functionally, suggesting, as depicted in Figure 3, that there are independent but interactive active sites. For example, treatment of native PGH synthase 1 with aspirin leads to concomitant acetylation of Ser530 and irreversible inactivation of cyclooxygenase activity without appreciably affecting peroxidase activity (van der Ouderaa et al., 1980; Mizuno et al., 1982); acetylation of Ser530 appears to prevent access of arachidonate to the cyclooxygenase active site (Smith et al., 1990). Several PGH synthase 1 mutants, including one with an asparagine in place of the serine at position 530, have also been found to lack cyclooxygenase but retain peroxidase activity (Smith et al., 1990; DeWitt et al., 1990). On the other hand, there are three forms of the enzyme which exhibit little or no peroxidase activity but which retain the ability to catalyze the cyclooxygenase reaction. These include (a) holoenzyme in which Fe^{3+} heme is replaced with Mn^{3+} heme (Ogino et al., 1978; Hemler et al., 1978a), (b) holoenzyme incubated in the presence of 250 mM NaCN (Hemler & Lands, 1980), and (c) a PGH synthase 1 mutant in which His386 (Figure 3) has been substituted with either an alanine or a glutamine residue (Shimokawa & Smith, 1991).

Elimination of cyclooxygenase activity with aspirin has no apparent effect on peroxidase catalysis; however, as depicted in Figure 4, reduction or elimination of the peroxidase activity has two significant effects on cyclooxygenase catalysis. One is to cause a pronounced lag period before maximal oxygen utilization is achieved, and a second is to reduce markedly the rate at which the cyclooxygenase becomes inactivated.

Cyclooxygenase Activation by Hydroperoxide. There is considerable evidence that the cyclooxygenase activity of PGH synthase 1 requires a peroxide to function. The cyclooxygenase is inhibited by glutathione peroxidase in the presence of glutathione, and this inhibition can be overcome by adding a hydroperoxide such as PGG_2 (Smith & Lands, 1972a,b;

† This work was supported in part by U.S. Public Health Service Grants DK42509 (W.L.S.), DK22042 (W.L.S.), GM30509 (R.J.K.), and CA47479 (L.J.M.) and a Grant-in-Aid from the American Heart Association of Michigan (W.L.S.).

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[1] PGH synthase 1 refers to the PGH synthase which has been cloned from mouse (DeWitt et al., 1989), human (Yokoyama & Tanabe, 1989; Funk et al., 1991), and ovine sources (DeWitt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988), has a signal peptide, and, after cleavage of the signal peptide, contains 576 amino acids. cDNAs coding for what appears to be another PGH synthase isozyme (PGH synthase 2) have recently been cloned from murine (Kujubu et al., 1991) and chicken (Xie et al., 1991) sources.

[2] Numbering of the amino acids begins with methionine at the translational start site.

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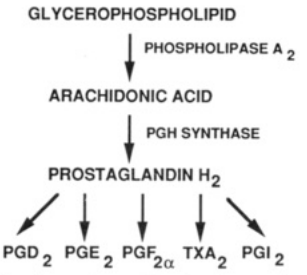

FIGURE 1: Biosynthetic pathway for the formation of various prostanoids.

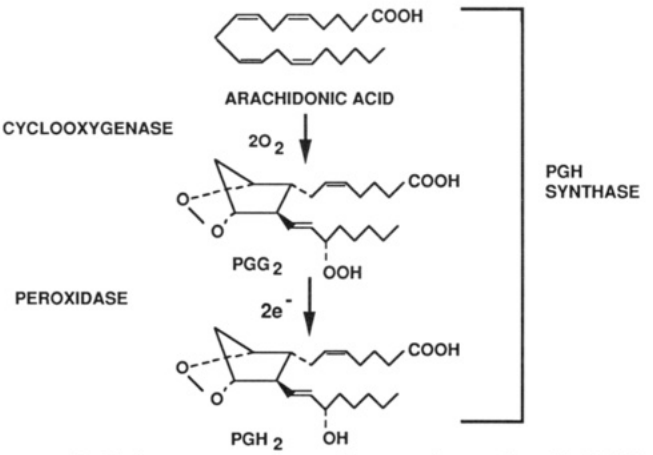

FIGURE 2: Cyclooxygenase and peroxidase reactions catalyzed by PGH synthase 1.

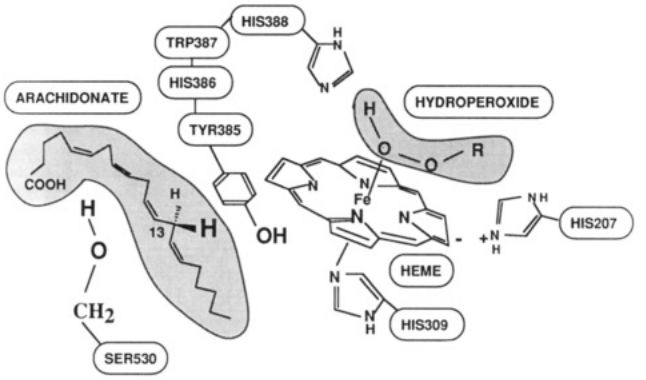

FIGURE 3: Model for the cyclooxygenase and peroxidase active sites of PGH synthase 1. Residues involved in heme binding and the relative locations of groups neighboring the arachidonate (cyclooxygenase) and hydroperoxide (peroxidase) binding sites are illustrated. Adapted from Shimokawa and Smith (1991).

Hemler et al., 1978b). Similarly, addition of a hydroperoxide to peroxidase-deficient forms of PGH synthase 1 eliminates the kinetic lag in the cyclooxygenase reaction (Hemler et al., 1978a; Hemler & Lands, 1980). The lag phase in attaining maximal cyclooxygenase activity can be viewed as a period in which there is insufficient hydroperoxide, typically in the form of PGG_2, to fully activate the cyclooxygenase. Apparently, small amounts of endogenous alkyl hydroperoxides contaminating preparations of arachidonate activate the first few cyclooxygenase reaction centers which then produce PGG_2. Newly formed PGG_2 activates other PGH synthase 1 molecules until all the cyclooxygenase is activated. In short, the cyclooxygenase must be activated and activation requires an oxidant hydroperoxide.

Mechanism of Hydroperoxide-Dependent Activation of Cyclooxygenase. The initial step in the cyclooxygenase reaction is removal of the 13-*pro-S* hydrogen from arachidonate (Hamberg & Samuelsson, 1967). An arachidonyl radical is

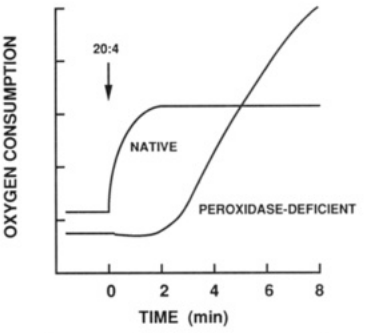

FIGURE 4: Schematic comparison of O_2 consumption profiles for the cyclooxygenase activities of native and peroxidase-deficient forms of PGH synthase 1.

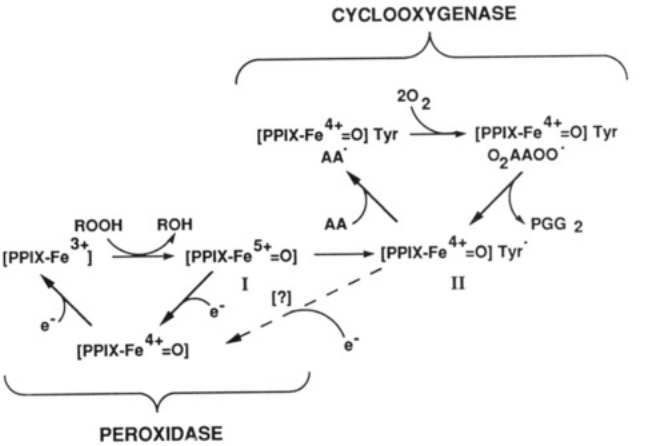

FIGURE 5: Model for peroxide-dependent activation of cyclooxygenase activity of PGH synthase 1 via formation of an intermediate tyrosyl radical: PPIX-Fe^{3+}, heme; AA, arachidonic acid. Adapted from Dietz et al. (1988).

formed when arachidonate and PGH synthase 1 are incubated anaerobically, and these results suggested that the 13-*pro-S* hydrogen is removed as a hydrogen atom (Mason et al., 1980; Schreiber et al., 1986). Further support for a free radical mechanism comes from findings of Fried and co-workers (Kwok et al., 1987) that PGH synthase 1 converts 10,10-difluoroarachidonate to (11*S*)-10,10-difluoro-11-hydroxy-5-(*Z*),8(*Z*),12(*E*),14(*Z*)-eicosatetraenoic acid (11-HETE) and epimeric 8,15-diHETEs but that no cyclization to prostaglandins occurs. This product profile can only be rationalized if the 13-*pro-S* hydrogen is abstracted from 10,10-difluoroarachidonate as an atom and not as a hydride or a proton.

The relationship between hydroperoxide activation of the cyclooxygenase and removal of the 13-*pro-S* hydrogen from arachidonic acid is of considerable interest. Recently, Ruf and co-workers proposed a very plausible model to describe this process (Dietz et al., 1988; Figure 5). According to this model, reaction of a hydroperoxide with the heme group at the peroxidase active site leads to formation of intermediate I; this occurs with concomitant reduction of the hydroperoxide to its corresponding alcohol. Intermediate I then abstracts a hydrogen from a neighboring tyrosine, producing a second oxidized heme species, intermediate II, containing a tyrosyl radical. Finally, the tyrosyl radical is envisioned as the active species which abstracts the 13-*pro-S* hydrogen from arachidonate to initiate the cyclooxygenase reaction.

A synopsis of the information supporting the Ruf model is as follows. First, it is well established that incubation of hydroperoxides with PGH synthase 1 leads to sequential formation of two distinct spectral intermediates with characteristics closely resembling compound I and compound ES peroxidase intermediates (Lambeir et al., 1985; Kulmacz et

al., 1987; Dietz et al., 1988; Hsuanyu & Dunford, 1990); these are denoted as intermediates I and II, respectively, in Figure 5. In addition, a tyrosyl radical, purportedly about 7–12 Å distant from the heme center, is formed rapidly in up to 80% yield based on heme content when hydroperoxides are incubated with native PGH synthase 1 (Karthein et al., 1988; Kulmacz et al., 1990).[3] Importantly, tyrosyl radical formation and formation of a compound ES like heme species, intermediate II, occur concomitantly (Dietz et al., 1988; Karthein et al., 1988). Fourth, modification of tyrosyl residues by tetranitromethane inhibits cyclooxygenase activity and alters the EPR spectrum of the tyrosyl radical (Smith et al., 1990; Kulmacz et al., 1990). And finally, there is compelling evidence that one tyrosine residue—Tyr385—is present at the cyclooxygenase active site of PGH synthase (Shimokawa et al., 1990; Smith et al., 1990; Figure 3).[4]

The Ruf model is attractive because it accounts for the peroxide dependence of the cyclooxygenase reaction, the formation of compound I and complex ES like ferryl–oxo species, the codependence of cyclooxygenase and peroxidase activities on heme, and the generation of a tyrosyl radical in the presence of hydroperoxide. To reiterate, the two central features of the model are (a) that peroxidase higher oxidation states are involved in cyclooxygenase activation and (b) that the tyrosyl radical is the species which abstracts the hydrogen from arachidonate to initiate the cyclooxygenase reaction.

Dependence of Cyclooxygenase Activation on Heme Oxidation. The preponderance of current evidence supports the idea that oxidized heme intermediates of peroxidase catalysis are involved in cyclooxygenase activation. First, these oxidized intermediates are formed rapidly in the presence of low concentrations of hydroperoxides required for cyclooxygenase activation (Lambeir et al., 1985). Second, the estimated K_d for hydroperoxide binding to PGH synthase (10 nM) is about the same as the concentration required for half-maximal cyclooxygenase activation (Kulmacz & Lands, 1983; Kulmacz, 1986). Third, the rate constants for oxidation of the synthase heme by different hydroperoxides parallel the effectiveness of the hydroperoxides in activating the cyclooxygenase (Kulmacz, 1991). And finally, the K_i value for inhibition of cyclooxygenase activation by cyanide ion is essentially the same as both the K_i for inhibition of the peroxidase and the K_d for cyanide binding to the heme (Kulmacz & Lands, 1985).

While the present evidence clearly favors the intermediacy of an oxidized heme species in cyclooxygenase activation, there are some data which are difficult to reconcile with this first part of the Ruf model. Specifically, no good correlation has been found between the efficiency with which reducing agents act as cosubstrates for the peroxidase reaction and the ability of these reducing substrates to inhibit the cyclooxygenase (Markey et al., 1987). For example, incubation of native PGH synthase 1 with concentrations of aryl sulfides which should have significantly reduced the levels of peroxidase-based oxidants did not affect cyclooxygenase activity (Plé & Marnett, 1989). Unfortunately, a confounding factor in interpreting the effects of reducing agents is the ability of some of these agents acting at low concentrations to stimulate the cyclooxygenase (Hemler & Lands, 1980; Egan et al., 1981). Further tests for the participation of oxidized heme intermediates in cyclooxygenase activation will likely involve comparing spectroscopic properties of the peroxidase-deficient forms of PGH synthase 1 with those of the native enzyme in the presence of low levels of alkyl hydroperoxide required for cyclooxygenase activation.

It is difficult to envision how heme could promote peroxide activation of the cyclooxygenase activity without the heme becoming oxidized.[5] However, heme likely plays at least one additional role in cyclooxygenase catalysis independent of its function as an oxidant. Specifically, heme binding causes structural changes in the protein which probably facilitate arachidonate binding to PGH synthase 1. PGH synthase 1 is sensitive to cleavage by trypsin, but only in the absence and not the presence of heme (Kulmacz & Lands, 1982; Chen et al., 1987); moreover, the rates of interaction of PGH synthase 1 with both aspirin and indomethacin are much faster with holoenzyme than apoenzyme (Chen & Marnett, 1989; Kulmacz, 1989). Because aspirin and indomethacin are competitive inhibitors of arachidonate binding (Rome & Lands, 1975), one would infer that heme facilitates arachidonate binding as well.

Role of a Tyrosyl Radical in the Cyclooxygenase Reaction. The second major proposition of the Ruf model is that a tyrosyl radical is involved in hydrogen atom abstraction from arachidonate (Figure 5). As noted earlier, it is clear that a tyrosyl radical is formed when PGH synthase 1 is incubated with either arachidonic acid or a hydroperoxide. Furthermore, at least one tyrosine residue—Tyr385—plays an integral role in cyclooxygenase catalysis.[4] However, some recent studies have failed to offer support for the direct involvement of a tyrosyl radical per se in cyclooxygenase catalysis and have raised the possibility that the observed tyrosyl radical could be associated with enzyme inactivation. Studies of Lassmann et al. (1991) have indicated that there is not a good correlation between cyclooxygenase catalysis and the appearance of the tyrosyl radical originally described by Karthein et al. (1988). Lassmann et al. (1991) observed that addition of phenol, a peroxidase-reducing cosubstrate, diminished the magnitude of arachidonate-dependent tyrosyl radical formation but without appreciably affecting cyclooxygenase product formation. Importantly, in the presence of 500 μM phenol, the first detectable tyrosyl radical signal was not observed until 60 s after PGH synthase 1 and its substrates were mixed and after completion of the cyclooxygenase reaction; with phenol, only the narrow singlet signal was observed; no doublet signal could be detected.[3] In related experiments with arachidonate,

[3] The issue of the tyrosyl radical is somewhat complicated. A doublet tyrosyl radical is formed rapidly when PGH synthase 1 is incubated with hydroperoxide or arachidonate (Karthein et al., 1988; Kulmacz et al., 1990; Lassmann et al., 1991); a second radical species, a broad singlet, appears subsequent to the doublet and probably represents a radical on the same tyrosine residue involved in the doublet signal; a third radical species, which appears much later, has not been well characterized.

[4] Tyr355, Tyr385, and Tyr417 residues of PGH synthase 1 are all nitrated by tetranitromethane, and all are protected from nitration in the absence but not the presence of indomethacin. Conversion of Tyr385 (Figure 3) to a phenylalanine by site-directed mutagenesis eliminates the cyclooxygenase but not the peroxidase activity of PGH synthase 1 (Shimokawa et al., 1990); however, mutants containing phenylalanine residues in place of either Tyr355 or Tyr417 retain considerable cyclooxygenase and peroxidase activity.

[5] In considering the necessity for peroxidase higher oxidation states in cyclooxygenase activation, the case of soybean lipoxygenase 1 is instructive. This enzyme, which contains a nonheme iron, converts arachidonic acid to 15-hydroperoxyeicosatetraenoic acid. The first step is the stereospecific removal of the 13-*pro-S* hydrogen, and the kinetic properties of this enzyme are similar to those of the cyclooxygenase activity of PGH synthase 1 (Smith & Lands, 1972). Both enzymes exhibit a pronounced lag which can be overcome by adding hydroperoxide; moreover, both enzymes undergo a suicide inactivation reaction with similar kinetic characteristics. Peroxide-dependent activation of lipoxygenase involves oxidation of the nonheme iron (Slappendel et al., 1982; Cheesbrough & Axelrod, 1983). Thus, there is at least one other mechanism known for peroxide-dependent activation of fatty acid oxygenases which does not involve heme.

they were also unable to detect any tyrosyl radical formation with the peroxidase-deficient, Mn^{3+}-heme PGH synthase 1 prior to the time that most of the cyclooxygenase was inactivated; furthermore, no radical signal was detected at any time after incubation of 5-phenyl-4-pentenyl 1-hydroperoxide with Mn^{3+}-heme-containing PGH synthase 1. In short, Lassmann et al. (1991) did not find a good correspondence between cyclooxygenase activity and either the magnitude of the tyrosyl radical signal or the time course of its appearance. Assuming a linear relationship between tyrosyl radical formation and arachidonate turnover, these findings suggest that the tyrosyl radical detected when PGH synthase 1 is incubated with hydroperoxides may *not* be directly involved in cyclooxygenase catalysis.[6]

Tyrosyl Radicals and Inactivation of PGH Synthase 1. If the tyrosyl radical is not involved in cyclooxygenase catalysis, what is its significance? One possibility is that this radical is formed as an intermediate at some point during the inactivation of PGH synthase 1. Native PGH synthase 1 undergoes suicide inactivation of both its peroxidase and cyclooxygenase activities during catalysis. This is depicted in Figure 4 for the cyclooxygenase activity as a fall in the rate of O_2 consumption prior to complete utilization of either O_2 or arachidonate substrates. The rate of loss of cyclooxygenase activity during incubation with arachidonate occurs at least 2 or 3 times faster than the rate of loss of peroxidase activity, suggesting that inactivation of the two activities occurs through different processes (Kulmacz, 1987).

As noted above, tyrosyl radical accumulation was diminished when holo-PGH synthase 1 and arachidonate were incubated in the presence of phenol, and the radical signal appeared after the loss of cyclooxygenase activity (Lassmann et al., 1991). These results raise the possibility that tyrosyl radical formation is associated with inactivation of the peroxidase activity of PGH synthase 1. Consistent with this possibility are findings which indicate that peroxidase reducing cosubstrates slow the rate of peroxidase inactivation (Kulmacz, 1986). A potential chemical precedent exits in the case of myoglobin. Peroxide-dependent oxidation of the heme group of myoglobin leads to generation of tyrosyl radicals which can react with the heme (Catalano et al., 1989). The availability of a variety of PGH synthase 1 mutants with tyrosine to phenylalanine substitutions (Shimokawa et al., 1990) may help identify that tyrosine or those tyrosines which are the source of the tyrosyl radical and clarify the relationship between radical formation and enzyme inactivation.

Summary. The initial step in the cyclooxygenase reaction catalyzed by PGH synthase 1 is abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid. The cyclooxygenase requires a peroxide to become activated. The peroxide probably oxidizes the heme group of PGH synthase 1 to a compound I like peroxidase intermediate as an integral part of the activation process. A radical species is then formed which abstracts the hydrogen from arachidonate. This species has not yet been identified. A tyrosyl radical is formed when PGH synthase 1 is incubated with arachidonate or peroxide, but evidence supporting a role for this radical in cyclooxygenase catalysis is not compelling; it is possible that the tyrosyl radical could be associated with suicide inactivation of the peroxidase activity of PGH synthase 1. The heme group of PGH synthase 1 probably plays an additional role in cyclooxygenase catalysis apart from its function in cyclooxygenase activation; specifically, heme probably acts allosterically to facilitate arachidonate binding.

[6] There may well be a radical, even a tyrosyl radical, involved in cyclooxygenase catalysis which remains below the micromolar level required for detection by EPR.

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