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Cyclooxygenase Inactivation Kinetics during Reaction of Prostaglandin H Synthase-1 with Peroxide†

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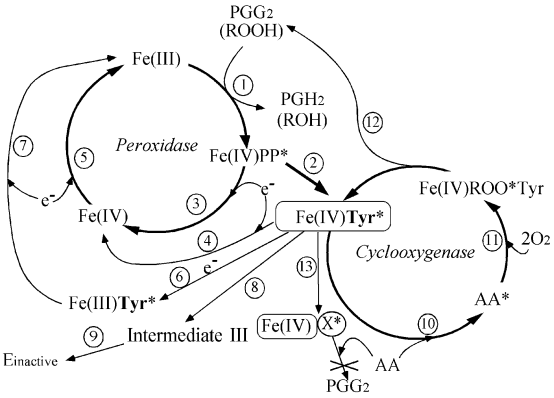
ABSTRACT: The peroxidase and cyclooxygenase activities of prostaglandin H synthase-1 (PGHS-1) both become irreversibly inactivated during reaction with peroxide. Sequential stopped-flow absorbance measurements with a chromogenic peroxidase cosubstrate previously were used to evaluate the kinetics of peroxidase inactivation during reaction of PGHS-1 with peroxide [Wu, G., et al. (1999) *J. Biol. Chem. 274*, 9231−7]. This approach has now been adapted to use a chromogenic cyclooxygenase substrate to analyze the detailed kinetics of cyclooxygenase inactivation during reaction of PGHS-1 with several hydroperoxides. 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. Cyclooxygenase inactivation kinetics appeared to be sensitive to the structure of the peroxide used. The addition of reducing cosubstrate during reaction of PGHS-1 with peroxide protected the peroxidase activity to a much greater degree than the cyclooxygenase activity. The results suggest a new concept of PGHS inactivation: that distinct damage can occur at the two active sites during side reactions of Intermediate II, which forms during reaction of PGHS with peroxide and which contains two oxidants, a ferryl heme in the peroxidase site, and a tyrosyl free radical in the cyclooxygenase site.

Prostaglandin H synthase (PGHS-1)[1] catalyzes the biosynthesis of the prostanoid, PGH_2, from arachidonic acid (AA). PGHS-1 has two enzymatic activities, a cyclooxygenase activity that converts AA to PGG_2 and a peroxidase activity that reduces PGG_2 to PGH_2 (*1*). Both activities require heme as a prosthetic group. Cyclooxygenase activity is generally dependent on peroxidase activity, but peroxidase activity is not disturbed by pharmacological or mutagenic treatments targeted at the cyclooxygenase activity (*2*, *3*). These experimental observations are well explained by the branched-chain reaction mechanism shown in Scheme 1. According to this mechanism, resting PGHS-1 [Fe(III)] reacts with a peroxide such as PGG_2 to generate Intermediate I [Fe(IV)PP*] (reaction 1), which converts via an intramolecular electron transfer (reaction 2) to Intermediate II [Fe(IV)Tyr*]. The latter species most likely contains a Tyr385 neutral radical because tyrosine cation radicals have pK_a values as low as −2 (*30*), and in PGHS the H-bonding of Tyr385 with adjacent Tyr348 and a structured water facili-

Scheme 1: Branched-Chain Radical Mechanism of PGHS-1, with Hypothetical Self-Inactivation Pathways[a]



[a] The mechanism is adapted from that originally proposed by Karthein et al. (*8*). Fe(III), resting enzyme with ferric heme; Fe(IV)PP*, Intermediate I with ferryl heme and porphyrin radical; Fe(IV)Tyr*, Intermediate II with ferryl heme and Tyr385 radical; AA*, arachidonic acid radical in cyclooxygenase site; Fe(IV)ROO*Tyr, PGG_2 radical in cyclooxygenase site; Fe(III)Tyr*, intermediate with ferric heme and Tyr385 radical; Fe(IV), intermediate with ferryl heme; ROOH and ROH, hydroperoxide and corresponding alcohol; e^-, reducing cosubstrate. Intermediate II is proposed to undergo damage either at the peroxidase site via step 8, leading to Intermediate III and terminal intermediate ($E_{inactive}$) (*6*), or at the cyclooxygenase site via step 13 to form a species [Fe(IV)X*] that has lost cyclooxygenase activity but retains peroxidase activity. X is an amino acid residue in the cyclooxygenase activity site.

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[1] Abbreviations: PGHS-1, prostaglandin H synthase isoform 1; Fe(IV)PP*, peroxidase intermediate containing a ferryl heme with a porphyrin π-cation radical (also known as Intermediate I or Compound I); Fe(IV)Tyr*, a species containing a ferryl heme and a tyrosyl radical (also known as Intermediate II); AA, arachidonic acid; AA*, arachidonic acid carbon-centered radical; PGG_2, prostaglandin G_2; PGG_2*, prostaglandin G_2 radical; 20:2, *cis*,*cis*-eicosa-11,14-dienoic acid; 15-HPETE; 15-hydroperoxyeicosatetraenoic acid; EtOOH, ethyl hydrogen peroxide; PGH_2, prostaglandin H_2; PPHP, *trans*-5-phenyl-4-pentenyl-1-hydroperoxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; PPA, *trans*-5-phenyl-4-penten-1-ol.

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tates rapid deprotonation (*31*). The cyclooxygenase reaction cycle starts with abstraction of a hydrogen atom from arachidonic acid (AA) by the tyrosyl radical of Intermediate II (reaction 10) to form a fatty acid radical (AA*). Subsequent combination with two oxygen molecules and rearrangement of the fatty acid backbone (reactions 11) generates the PGG_2 radical (PGG_2*). Transferring a hydrogen atom from Tyr385 to the PGG_2 radical regenerates Intermediate II and releases PGG_2 (reaction 12), completing the cyclooxygenase catalytic cycle. Resting enzyme can be regenerated by reducing Intermediates I or II with endogenous or exogenous cosubstrates (e^- in reactions 3−7).

Both the peroxidase activity and the cyclooxygenase activity of PGHS undergo irreversible inactivation during catalysis (*1*, *4*), imposing an upper limit on the synthesis of potent prostanoid signaling molecules (*5*). Because Intermediate II has two kinds of oxidizing equivalents, Fe(IV) heme and the tyrosyl radical, abortive oxidative side reactions from Intermediate II presumably could cause damage at either the peroxidase site [from the Fe(IV) heme] or the cyclooxygenase site (from the tyrosyl radical). Loss of peroxidase activity would be expected to result from damage at the peroxidase site but not from damage to the cyclooxygenase site; after all, cyclooxygenase inhibitors do not alter peroxidase activity (*2*). However, cyclooxygenase activity could be lost upon damage either to the peroxidase site (because of the resulting inability to generate Intermediate II) or to the cyclooxygenase site itself.

Comparison of the rates of peroxidase and cyclooxygenase inactivation during reaction of PGHS with peroxide should reveal the relative rates of damaging events at the peroxidase and cyclooxygenase sites from Intermediate II. Our earlier studies used sequential stopped-flow reactions to analyze the kinetics of peroxidase inactivation during reaction of PGHS-1 with peroxide (*6*, *7*). The present studies extend this approach to analyze the kinetics of cyclooxygenase inactivation under the same conditions. Exogenous cosubstrates were omitted from most reactions to maximize accumulation of Intermediate II, thus facilitating characterization of inactivation processes originating with this key oxidized enzyme intermediate. Multiple peroxidase catalytic turnovers are sustained by endogenous reductant (*29*) under these conditions. The results indicate that the rate of cyclooxygenase inactivation is the same as, or somewhat faster than, the rate of peroxidase inactivation when PGHS-1 is reacted with various peroxides in the absence of exogenous cosubstrate, indicating that most of the damage from Intermediate II side reactions occurs at the peroxidase site. Reaction of PGHS-1 with peroxide in the presence of cosubstrate produced substantial inactivation of the cyclooxygenase but much less peroxidase inactivation, confirming the existence of selective cyclooxygenase damage(s) originating from peroxidase cycle intermediates. This new approach to analysis of cyclooxygenase inactivation kinetics opens the way to detailed comparison of self-inactivation in the two PGHS isoforms.

EXPERIMENTAL PROCEDURES

Materials. Hemin was purchased from either Sigma (St. Louis, MO) or Porphyrin Products (Logan, UT). EtOOH was purchased as a 5% aqueous solution from Polysciences Inc. (Warrington, PA), and its concentration was determined from the absorbance at 230 nm by use of an extinction coefficient of 43 mM^{-1} cm^{-1}. PPHP was purchased from Cayman Chemical Co. (Ann Arbor, MI). 15-HPETE was prepared from AA and soybean lipoxygenase (*9*). The purity of 15-HPETE was assessed chromatographically and its concentration was quantified from the extent of TMPD (Sigma) oxidation catalyzed by excess PGHS-1 by use of an extinction coefficient of 27 [mM peroxide reduced]$^{-1}$ cm^{-1} (*10*). Tween 20 was from Pierce (Rockford, IL) or Anatrace (Maumee, OH). Cholic acid (Sigma) was recrystallized from hot ethanol and prepared as a 20% aqueous stock solution. PGHS-1 was prepared as the apoenzyme from ram seminal vesicles, with 5 mM glutathione included in the isoelectric focusing step (*11*). PGHS-1 holoenzyme was prepared by adding excess heme to the apoenzyme; treating with DEAE-cellulose (DE52, Whatman) equilibrated with 100 mM potassium phosphate, pH 7.2; and chromatographing on a Bio-Rad 10-DG column (*12*). The concentration of PGHS-1 holoenzyme was based on the absorbance at 410 nm (165 mM^{-1} cm^{-1}) (*13*). Cyclooxygenase activity was assayed polarographically at 30 °C (*11*). The specific activity of PGHS-1 preparations used in the present study was ≥100 μmol of O_2 min^{-1} mg^{-1}.

Determination of PGHS-1 Peroxidase and Cyclooxygenase Inactivation Kinetics. Kinetic studies were conducted on a DX-18MV Bio-Sequential stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, U.K.). The instrument was equipped with an improved xenon lamp having increased light intensity in the UV, a high-sensitivity photomultiplier tube, and a 2 mm path length cell to enhance the signal-to-noise ratio at short wavelengths and keep absorbance within the linear range. PGHS-1 (0.1−2.0 μM) was mixed with peroxide (EtOOH, PPHP, or 15-HPETE) in the first stage, aged for 0.1−10 s, and then mixed in the second stage with 880 μM 20:2 in 0.1 M Tris buffer, pH 8.0, containing 10% glycerol, 0.1% Tween 20, and 0.05% cholate. Inclusion of the detergents help dissolve the fatty acid, thereby avoiding the previously reported initial drop in A_{235} (*14*). Enzyme and peroxide stocks were prepared in the same buffer without cholate. The surviving cyclooxygenase activity was quantified in the second-stage reaction from the rate of A_{235} increase due to formation of conjugated diene upon oxygenation of 20:2 (*14*, *15*). AA was not suitable for these studies because PGG_2 and PGH_2 lack a usable chromophore. To measure surviving peroxidase activity, a mixture of 10 mM guaiacol and 10 mM H_2O_2 in H_2O was used in the second stage, and oxidation of guaiacol was monitored at 436 nm (*6*, *7*).

Data Processing. Surviving cyclooxygenase activity was calculated from the maximal rate of fatty acid diene formation, obtained by a 15-point moving average smoothing of the first derivative of the A_{235} kinetic data. To compensate for the rapid enzyme inactivation, the first-derivative values were extrapolated back to zero time (in the second-stage reaction). Surviving peroxidase activity was calculated from the initial velocity of guaiacol oxidation in the second-stage reaction. Values for surviving cyclooxygenase or peroxidase activity measured in the second-stage reaction were plotted as a function of the aging time (first-stage reaction) and the rate constant for cyclooxygenase or peroxidase inactivation was obtained by fitting the data to

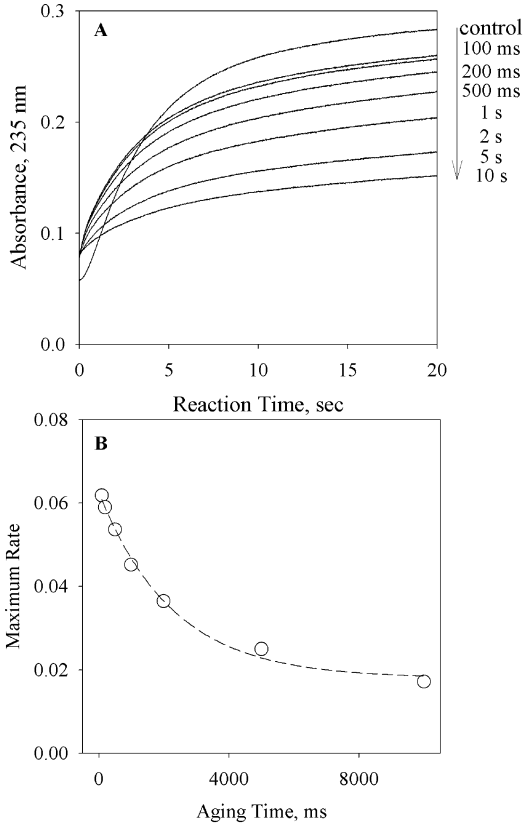

FIGURE 1: Kinetics of cyclooxygenase inactivation during reaction of PGHS-1 with peroxide. (A) Time courses from second-stage oxygenase reactions with 20:2, monitored by conjugated diene formation at 235 nm, for samples of PGHS-1 (1.64 μM) reacted for the indicated times in the first stage with PPHP (67.2 μM). The control reaction profile was obtained by mixing PGHS-1 with buffer in the first stage. (B) Oxygenase rates calculated from the data in panel A (○) are shown as a function of the aging time in the first-stage reaction with PPHP. The line represents an exponential decay function fitted to the data ($k = 0.44 \pm 0.05$ s^{-1}). The units of the ordinate are ΔA_{235} per second.

$$y = ae^{-bt} + y_\infty \quad (1)$$

where a is the initial activity susceptible to inactivation, b is the observed decay rate, t is the aging time, and y_∞ is the background activity at infinite aging time.

RESULTS

Kinetics of Cyclooxygenase Inactivation during Reaction of PGHS-1 with Peroxide. A set of second-stage 20:2 oxygenation kinetics data obtained at different aging times for a first-stage reaction of 1.64 μM PGHS-1 with 67.2 μM PPHP is presented in Figure 1. The lower initial absorbance in the control reaction (enzyme mixed with buffer only in the first stage) reflects the absence of PPHP, which absorbs at this wavelength; the initial lag phase is due to the initial lack of peroxide activator carried over from the first stage, necessitating accumulation of endogenous activator in the second stage. With peroxide included in the first stage, there was no lag, and the surviving cyclooxygenase activity for each first-stage aging time was readily calculated from the initial slope of the corresponding A_{235} kinetic data (Figure 1A). When the surviving cyclooxygenase activity values are plotted as a function of first-stage aging time (Figure 1B), it is apparent that reaction of PGHS-1 with peroxide in the first stage produced an exponential decay in the cyclooxygenase activity, here with a rate constant of 0.44 s^{-1}.

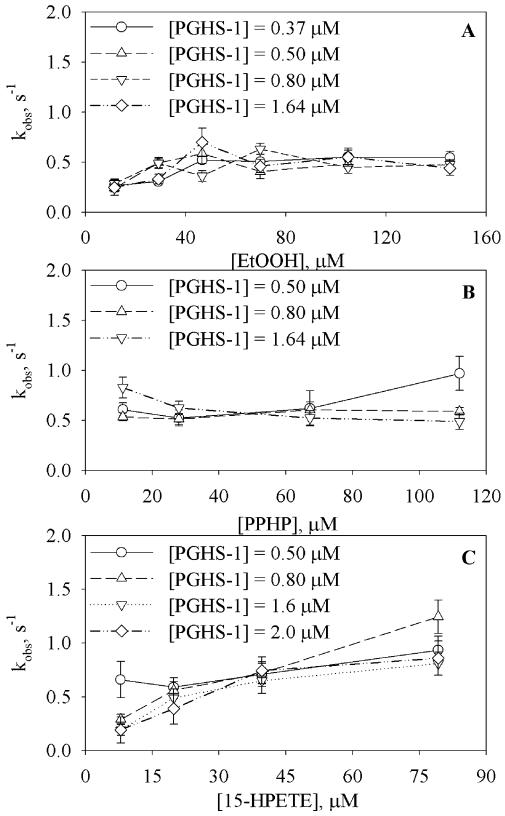

FIGURE 2: Effects of peroxide concentration and structure and PGHS-1 concentration on the rate of cyclooxygenase inactivation. Cyclooxygenase inactivation rates (k_{obs}) were determined at the indicated levels of EtOOH (A), PPHP (B), or 15-HPETE (C), at three or four different PGHS-1 concentrations, by the two-stage stopped-flow procedure described under Experimental Procedures. Each point represents the average of two or three determinations, with the error bar showing the standard deviation of the exponential fit to activity decay data.

The dependence of the rate of peroxide-induced cyclooxygenase inactivation on peroxide structure and concentration as well as enzyme concentration was also examined (Figure 2). In each case, the peroxide was reacted with PGHS-1 for various lengths of time in the first stage before measurement of the surviving cyclooxygenase activity in the second-stage reaction, as described above. For all three peroxides tested, the cyclooxygenase inactivation rate did not seem to depend on enzyme concentration (Figure 2). There were some outliers at the extremes of peroxide concentration, but these outliers were infrequent (only 4 of 52 data points in Figure 2) and did not show a systematic dependence on enzyme concentration, and are thus likely to represent data fluctuation. Accordingly, the inactivation rates obtained with the several enzyme levels at a given concentration of peroxide were averaged so that the dependence on peroxide level could be examined more easily for comparison with peroxidase inactivation rates obtained previously (*6*) (Figure 3). To ensure that current PGHS-1 preparations behaved similarly to those used in the previous study, we checked the peroxidase inactivation kinetics of several current enzyme preparations, using EtOOH, PPHP, or 15-HPETE in the first-stage reaction and a mixture of H_2O_2 and guaiacol (instead of 20:2) in the second-stage reaction, as was done in our earlier study (*6*) The resulting peroxidase inactivation rates

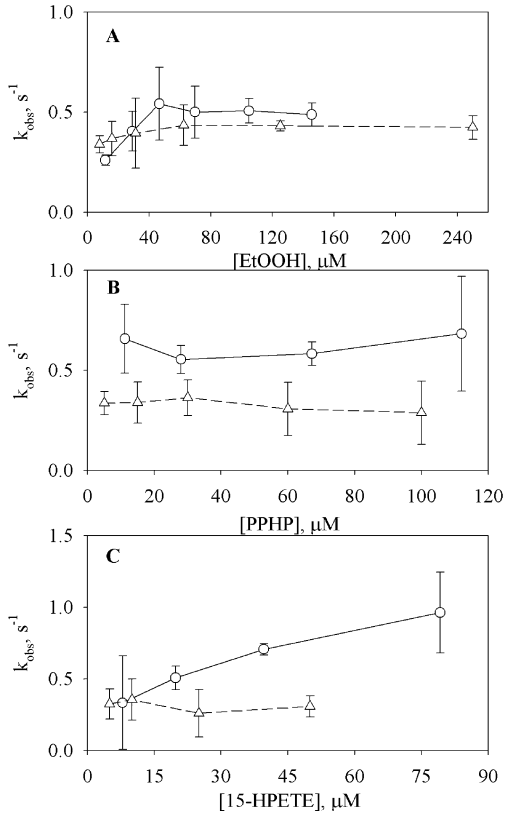

FIGURE 3: Comparison of the kinetics of peroxidase and cyclooxygenase inactivation during reaction of PGHS-1 with peroxides. Inactivation rates for cyclooxygenase (○; data from Figure 2) and peroxidase (△; data from ref 6) measured at different PGHS-1 concentrations were averaged and plotted as a function of peroxide concentration for EtOOH (A), PPHP (B), and 15-HPETE (C). The error bars indicate the standard deviations of the mean.

were in the range of 0.4−0.5 s^{-1}, very similar to the values obtained previously (data not shown).

With EtOOH or PPHP, the cyclooxygenase inactivation rate showed little or no significant change as the peroxide concentration was increased (Figure 3A,B). In the case of 15-HPETE, the cyclooxygenase inactivation rate did show a trend toward increasing with increasing peroxide concentration (Figure 3C).

There was no significant difference between cyclooxygenase and peroxidase inactivation rates for EtOOH (Figure 3A), but for PPHP and for higher levels of 15-HPETE the cyclooxygenase activity appeared to be lost more quickly than the peroxidase activity (Figure 3B,C).

Effects of Peroxidase Cosubstrate on Peroxidase and Cyclooxygenase Inactivation during Reaction of PGHS-1 with Peroxide. The effect of exogenous cosubstrate on peroxidase and cyclooxygenase inactivation rates was examined by including 500 μM guaiacol in the first-stage reaction along with EtOOH (Figure 4). In the absence of exogenous cosubstrate, both peroxidase and cyclooxygenase activities exhibited a fast initial decay (∼0.7 and 0.9 s^{-1}, respectively) followed by a slower decay phase (∼0.03 s^{-1}). Less than 10% of peroxidase activity and less than 20% of cyclooxygenase activity remained after 120 s of reaction with EtOOH. When 500 μM guaiacol was included in the reaction with EtOOH, about 90% of the peroxidase activity, but only about 50% of the cyclooxygenase activity, remained after 120 s (Figure 4). This result indicates that the presence of

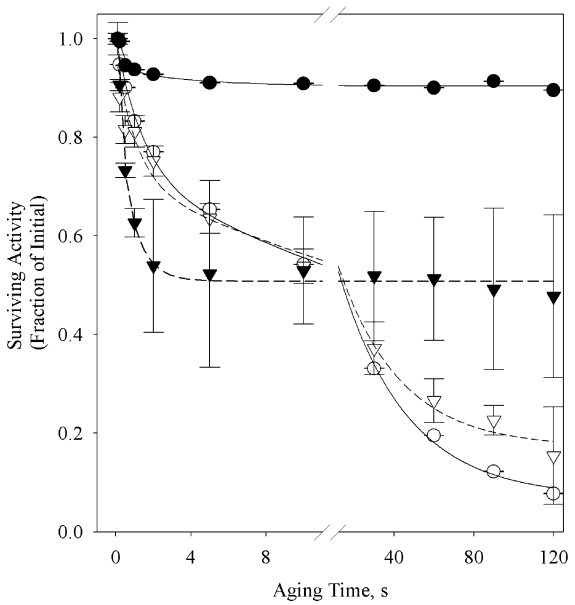

FIGURE 4: Effect of guaiacol on peroxide-induced inactivation of peroxidase and cyclooxygenase activities in PGHS-1. PGHS-1 (2.0 μM) was reacted in the first stage with EtOOH (60 μM) either with (solid symbols) or without (open symbols) 500 μM guaiacol for the indicated aging time before assay, in the second stage, of surviving peroxidase (with 10 mM guaiacol/10 mM H_2O_2; circles) or cyclooxygenase (with 400 μM 20:2; triangles) activity. Lines represent biphasic exponential fittings.

the cosubstrate had very different effects on cyclooxygenase and peroxidase inactivation, leading to preferential sparing of the peroxidase during reaction of PGHS-1 with peroxide. Neither activity was further damaged after about 5 s of reaction, presumably because of rapid depletion of the peroxide by peroxidase catalysis in the presence of cosubstrate at the elevated PGHS-1 level used in this experiment. Curiously, the presence of cosubstrate actually seemed to accelerate inactivation of the cyclooxygenase activity in the early part of the reaction; the explanation for this is unclear.

DISCUSSION

Crystallographic data show that the peroxidase and cyclooxygenase sites of PGHS-1 occupy structurally distinct, though adjacent, regions of the catalytic domain, with heme marking the peroxidase site and Tyr385 a landmark of the cyclooxygenase site (*16*). Both catalytic activities of PGHS-1 are irreversibly lost when the protein is reacted with peroxide (*17*).

Peroxidase catalysis involves initial reduction of the peroxide to alcohol and concomitant oxidation of the PGHS heme to form Intermediates I and II, followed by reduction of the enzyme back to resting state by reaction with cosubstrate (Scheme 1). Inactivation of the peroxidase presumably involves some sort of damage at the peroxidase site, which prevents further redox interactions with peroxide or cosubstrate. Self-inactivation in other peroxidases differs in many ways from PGHS peroxidase inactivation. PGHS peroxidase inactivation is not peroxide concentration-dependent (*6*), whereas a hyperbolic peroxide concentration dependence was found for other plant peroxidases (*32*, *33*). Thus, self-inactivation of plant peroxidases originates from Compound I (or Intermediate I), whereas the branching point of peroxidase inactivation from PGHS catalysis is at

Intermediate II. Our earlier rapid-scan stopped-flow studies of PGHS peroxidase catalysis and self-inactivation (*6*) did not detect formation of Compound III (oxyferrous heme), with its distinctive absorbance peaks at 419, 545, and 579 nm (*25*). In contrast, Compound III was found to be an important intermediate in plant peroxidase inactivation process in the presence of excess hydroperoxide (*33*). PGHS Intermediate II is important to both peroxidase and cyclooxygenase catalysis but does have two potentially damaging oxidants, a ferryl heme and a free radical on Tyr385. Tyr385 is not physically part of the peroxidase site and this tyrosine can be mutated to other residues without marked effect on peroxidase catalysis or inactivation (*3*, *18*), so damage from the Tyr385 radical in Intermediate II is unlikely to be the cause of peroxidase inactivation. Heme itself is not covalently modified during peroxidase inactivation (*6*), ruling out damage to the prosthetic group as the basis for loss of peroxidase catalytic competence. Peroxidase inactivation is thus most likely to involve some sort of protein damage in the peroxidase site by oxidant originating from the ferryl heme.

Cyclooxygenase catalysis is more complex than peroxidase catalysis. In the current branched-chain mechanism (Scheme 1), the cyclooxygenase remains latent until activated by peroxide-induced generation of a free radical on Tyr385. The Tyr385 radical abstracts a hydrogen atom from bound arachidonate to generate an arachidonate radical, which in turn attacks molecular oxygen and cyclizes to form a PGG_2 radical. Finally, a hydrogen atom transfer forms PGG_2 itself and regenerates the Tyr385 radical. Inactivation of the cyclooxygenase during reaction of PGHS-1 with peroxide might plausibly come from two sources: damage preventing the reactions with peroxide needed to generate the Tyr385 radical, or damage to the cyclooxygenase site preventing proper arachidonate binding or reaction of the fatty acid with the Tyr385 radical. In principle, cyclooxygenase catalysis is thus vulnerable to peroxide-driven protein damage either at the peroxidase site (with ferryl heme as the oxidant) or at the cyclooxygenase site (with the Tyr385 radical as the oxidant). With two potential routes to cyclooxygenase inactivation and only one route to peroxidase inactivation, this conceptual analysis predicts that the rate of cyclooxygenase loss will be equal to or greater than that for peroxidase loss when PGHS-1 is reacted with peroxide. This prediction is in fact borne out by the present results (Figure 3), where cyclooxygenase activity appeared to be lost more quickly than peroxidase activity at some levels of two of the three peroxides examined. The greater inactivation of cyclooxygenase than peroxidase during reaction with peroxide was dramatically confirmed in reactions with cosubstrate present (Figure 4).

Any increment of the cyclooxygenase inactivation rate over that for peroxidase inactivation is modest (Figure 3), suggesting comparable rates for damage to the peroxidase and cyclooxygenase sites. The fact that a similar increment in cyclooxygenase inactivation over peroxidase inactivation was not observed for all peroxides suggests that some aspect of cyclooxygenase inactivation is sensitive to the peroxide structure. This behavior is not predicted by the mechanism in Scheme 1, which has the inactivation events occurring after reaction of PGHS-1 with peroxide. However, unexpected sensitivity to peroxide structure also was observed in the rate constant for generation of Intermediate II (reaction 2 in Scheme 1) (*19*). It may be that these structural effects reflect continued binding of lipophilic alcohol product (i.e., PPA and 15-HETE), which is not seen with the small, hydrophilic product (EtOH). In this interpretation, the bound lipophilic alcohol alters the properties of the tyrosyl radical in Intermediate II so as to enhance cyclooxygenase site damage. Another possibility is that the lipophilic peroxides bind directly to, and damage, the cyclooxygenase site in second-order reactions. Direct damage by peroxides at the cyclooxygenase site might also account for the concentration dependence of cyclooxygenase inactivation by 15-HPETE (Figure 3C).

The present studies are the first to directly quantify the detailed kinetics of cyclooxygenase inactivation during reaction of PGHS-1 with peroxide. However, several earlier studies did compare inactivation of cyclooxygenase and peroxidase after fixed-time incubations with peroxide. In the earliest study (*17*), a 2−3 min incubation with 15-HPETE in the absence of cosubstrate produced comparable inactivation of the two activities except at higher peroxide levels, where slightly more peroxidase activity survived. In later titrations of PGHS-1 with increasing amounts of either HOOH or EtOOH in the absence of added cosubstrate, somewhat more cyclooxygenase than peroxidase activity was inactivated throughout the process (*20*). More recently, 5-min incubations of PGHS-1 with various levels of EtOOH in the presence of cosubstrate resulted in comparable loss of cyclooxygenase and peroxidase activities except at the lowest peroxide level, where considerably more peroxidase activity survived (*18*). The results from each of these "static" studies are qualitatively similar to those from the present "kinetic" studies, with the cyclooxygenase being inactivated to the same or a somewhat greater extent than the peroxidase activity.

Results from the present examination of peroxidase and cyclooxygenase inactivation kinetics during PGHS reaction with peroxide will no doubt be useful for analyzing inactivation of the two activities during reaction with fatty acid substrate. The latter case is more complex because cyclooxygenase and peroxidase catalysis occur simultaneously and there is the additional potential for cyclooxygenase inactivation by damage from potentially reactive lipid intermediates in the cyclooxygenase catalytic cycle, such as fatty acid radicals (*21*). This additional inactivation mode presumably underlies the variation in cyclooxygenase catalytic turnovers with different fatty acid substrates (*17*, *22*) and can be seen at its most extreme in the case of acetylenic fatty acids, which act as suicide substrates (*23*).

PGHS catalysis in vivo occurs in the presence of a variety of small reductant molecules, such as uric acid, which act as cosubstrates for the peroxidase activity (*24*). These "exogenous" peroxidase cosubstrates are recognized to reduce oxidized enzyme intermediates generated during reaction with peroxide, returning the enzyme to resting state without altering the nature of the oxidized enzyme intermediates (*25*). The goal of the present studies was to analyze the kinetics of inactivating events that originate from the oxidized peroxidase intermediates and so the concentrations of these intermediates were maximized by not adding exogenous cosubstrate in most experiments. The same experimental tactic of omitting exogenous cosubstrate has

proved indispensable for the characterization the oxidized intermediates themselves by optical and electron paramagnetic resonance (EPR) spectroscopy (*8*, *12*, *25*, *26*). In particular, the tyrosyl radical in Intermediate II, which is established as the crucial oxidant in cyclooxygenase catalysis, is readily studied in the absence of exogenous cosubstrate but is difficult to detect if appreciable levels of reductant are added (*27*). Omitting exogenous cosubstrate has the additional advantage of avoiding complexities due to the large variations among cosubstrates in the rates of reaction with oxidized enzyme intermediates (*17*, *28*) and in the rates of potentially damaging reactions with oxidized cosubstrate. The latter may account for the faster initial phase of inactivation observed in the presence of cosubstrate in Figure 4. It should be noted that purified PGHS-1 itself contains some "endogenous" reductant (*20*, *29*). Thus, a limited number of peroxidase catalytic cycles occur even in the absence of added cosubstrate. The simplest interpretation of currently available data is that cosubstrates attenuate inactivation of the peroxidase and cyclooxygenase activities primarily by decreasing accumulation of the oxidized enzyme intermediates that undergo the damaging side reactions, rather than by fundamental changes in the inactivation processes. Systematic characterization of the effects of cosubstrate concentration and structure on inactivation kinetics will be useful in testing this interpretation.

In summary, reaction of PGHS with peroxide appears to damage both the peroxidase and cyclooxygenase active sites, consistent with the presence of distinct, reactive oxidants at the heme and at Tyr385 in Intermediate II, with the relative rate of damage to the two sites dependent on peroxide structure and the presence of reducing cosubstrates.

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