Comparison of the Peroxidase Reaction Kinetics of Prostaglandin H Synthase-1 and -2*

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Prostaglandin H synthase isoforms 1 and 2 (PGHS-1 and -2) each have a peroxidase activity and also a cyclooxygenase activity that requires initiation by hydroperoxide. The hydroperoxide initiator requirement for PGHS-2 cyclooxygenase is about 10-fold lower than for PGHS-1 cyclooxygenase, and this difference may contribute to the distinct control of cellular prostanoid synthesis by the two isoforms. We compared the kinetics of the initial peroxidase steps in PGHS-1 and -2 to quantify mechanistic differences between the isoforms that might contribute to the difference in cyclooxygenase initiation efficiency. The kinetics of formation of Intermediate I (an Fe(IV) species with a porphyrin free radical) and Intermediate II (an Fe(IV) species with a tyrosyl free radical, thought to be the crucial oxidant in cyclooxygenase catalysis) were monitored at 4°C by stopped flow spectrophotometry with several hydroperoxides as substrates. With 15-hydroperoxyeicosatetraenoic acid, the rate constant for Intermediate I formation ($k_1$) was 2.3 × 10$^4$ M$^{-1}$ s$^{-1}$ for PGHS-1 and 2.5 × 10$^4$ M$^{-1}$ s$^{-1}$ for PGHS-2, indicating that the isoforms have similar initial reactivity with this lipid hydroperoxide. For PGHS-1, the rate of conversion of Intermediate I to Intermediate II ($k_2$) became the limiting factor when the hydroperoxide level was increased, indicating a rate constant of 10$^2$–10$^3$ s$^{-1}$ for the generation of the active cyclooxygenase species. For PGHS-2, however, the transition between Intermediates I and II was not rate-limiting even at the highest hydroperoxide concentrations tested, indicating that the $k_2$ value for PGHS-2 was much greater than that for PGHS-1. Computer modeling predicted that faster formation of the active cyclooxygenase species (Intermediate II) or increased stability of the active species increases the resistance of the cyclooxygenase to inhibition by the intracellular hydroperoxide scavenger, glutathione peroxidase. Kinetic differences between the PGHS isoforms in forming or stabilizing the active cyclooxygenase species can thus contribute to the difference in the regulation of their cellular activities.

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†† The abbreviations used are: PGHS, prostaglandin H synthase; PGG$_2$, prostaglandin G$_2$; EOOH, ethyl hydrogen peroxide; 15-HPETE, 15-hydroxy-peroxyeicosatetraenoic acid; PPHP, trans-5-phenyl-4-pentenyl-1-hydroperoxide; GSP, glutathione peroxidase.
whether the porphyrin is in ground (Intermediate) or has a tyrosyl radical present (Tyr*). $E_{inact}$ represents self-inactivated enzyme, ROOH and ROH are hydroperoxide and the corresponding alcohol, $e^-$ is an electron donor (reducing cosubstrate), and AA is arachidonic acid.

### MATERIALS AND METHODS

Glutathione, GSP, glycerol, hemin chloride, and Pharmalytes (pH 5–8) were from Sigma. PD-10 desalting columns were from Supelco. Tween 20 was obtained routinely used without borohydride treatment. Ticlopidine, PGHS-2, 15-HPETE, and Tyr371 in PGHS-2) is in ground state (Tyr), $E_{(III)/PPIX/Tyr}$ or has a tyrosyl radical present (Tyr*).

### PROSTAGLANDIN H SYNTHASE-1 AND -2 PEROXIDASE KINETICS

#### SCHEME I. Branched chain radical mechanism for PGHS peroxidase and cyclooxygenase catalysis based on a proposal by Ruf and colleagues (7). The letters to the right side of each enzyme intermediate indicate the redox state of the heme iron (III or IV), whether the porphyrin is in ground (Intermediate) or has a tyrosyl radical present (Tyr*).

#### Table I. Parameter values used for kinetic simulations

| Parameter | Value | Units |
|-----------|-------|-------|
| $k_1$     | $1 \times 10^8$ | M$^{-1}$s$^{-1}$ |
| $k_2$     | 350–8750 | M$^{-1}$s$^{-1}$ |
| $k_3$     | $4 \times 10^3$ | M$^{-1}$s$^{-1}$ |
| $k_4$     | $4 \times 10^3$ | M$^{-1}$s$^{-1}$ |
| $k_5$     | 60 | M$^{-1}$s$^{-1}$ |
| $k_6$     | $2 \times 20^3$ | M$^{-1}$s$^{-1}$ |
| $k_7$     | $6 \times 10^{-2}$ | M$^{-1}$s$^{-1}$ |
| $K_{mAA}$ | 3 $\times 10^{-6}$ | M |
| $R_{GC}$  | 0–600 | M |
| $k_{GSP}$ | $1.5 \times 10^{-6}$ | M |
| $E/(III)/PPIX/Tyr$ | $1 \times 10^{-8}$ | M |
| $E/(IV)/PPIX/Tyr$ | 0 | M |
| $E/(IV)/PPIX/Tyr$ | 0 | M |
| $E/(III)/PPIX/Tyr$ | 0 | M |
| $E/(IV)/PPIX/Tyr$ | 0 | M |
| $E/(III)/PPIX/Tyr$ | 0 | M |
| $E/(IV)/PPIX/Tyr$ | 0 | M |
| $E/inact_0$ | 0 | M |
| $AA_0$ | $1 \times 10^{-4}$ | M |
| ROOH | $2 \times 10^{-8}$ | M |
| ROH | 0 | M |
| AH$_2$ | $5 \times 10^{-4}$ | M |

#### Equation 1

$$-k_5[E/(IV)/PPIX*/Tyr] - k_6[AH][E/(IV)/PPIX*/Tyr]$$

#### Equation 2

$$d[E/(IV)/PPIX*/Tyr]/dt = k_5[AH][E/(IV)/PPIX*/Tyr]$$

#### Equation 3

$$-k_7[AH][E/(IV)/PPIX*/Tyr]$$

#### Equation 4

$$d[E/(III)/PPIX*/Tyr]/dt = -k_6[ROOH][E/(III)/PPIX*/Tyr]$$

#### Equation 5

$$-k_4[AH][E/(IV)/PPIX*/Tyr]$$

#### Equation 6

$$d[E/(IV)/PPIX*/Tyr]/dt = -k_6[ROOH][E/(IV)/PPIX*/Tyr]$$

#### Equation 7

$$d[E/(III)/PPIX*/Tyr]/dt = -k_6[ROOH][E/(III)/PPIX*/Tyr]$$

#### Equation 8

$$d[E/(IV)/PPIX*/Tyr]/dt = -k_6[ROOH][E/(IV)/PPIX*/Tyr]$$

#### Equation 9

$$d[E/(III)/PPIX*/Tyr]/dt = -k_6[ROOH][E/(III)/PPIX*/Tyr]$$

In this model, cyclooxygenase activity is a simple, saturable function (the $K_\text{mAA}$ value for arachidonic acid is $K_{\text{mAA}}$) reflecting the rate constant ($k_5$), the arachidonate concentration ([AA]), and the total concentration of AA.
enzyme forms with activated cyclooxygenase (E(III)/PPIX/Tyr••, E(IV)/PPIX*/Tyr, and E(IV)/PPIX/Tyr••). The term for cyclooxygenase activity appears in Equations 8 and 9. Similarly, the glutathione peroxidase activity is a simple, saturable function reflecting the peroxide level ([ROOH]), the $k_m$ value for hydroperoxide, PGG2 ($K_m$(GGP)), and the GSP activity (calculated from the ratio of added GSP units to added cyclooxygenase units, $R_{GSP}$), the cyclooxygenase specific activity ($k_c$), and the initial PGHS concentration [E(III)/PPIX/Tyr••]). The term for GSP activity appears in Equations 9 and 10. The initial concentrations and parameter values for Equations 1–11 are shown in Table I. The value of $k_b$ was based on measured values for the rate of Intermediate I formation with lipid hydroperoxides (19). A range of $k_b$ values was tested, with the lower bound being the observed value for conversion of Intermediate I to Intermediate II in PGHS-1 (“Results” and Ref. 19). The rates for reduction of higher oxidation states of heme ($k_1$ and $k_2$) were consistent with the measured value for the overall rate of return to ferric heme with phenolic reducing cosubstrates (20). The value of $k_b$ represents the turnover number calculated from a PGHS-1 cyclooxygenase specific activity of 100 μmol O₂/min/mg protein. A range of $k_b$ values were tested, as described under “Results.” The value of $k_b$ was set at $10^{-7}$ times that of $k_c$ to fit the typical observation of about 1000 cyclooxygenase catalytic events before self-inactivation of PGHS-1 (21). Measured values were used for the cyclooxygenase $K_m$ for arachidonate ($K_{mac}$) and for the GSP $K_m$ for PGG2 ($K_{GGP}$) (21, 22).

RESULTS

Reaction of PGHS-1 and -2 with EtOOH—Reaction of PGHS-1 or PGHS-2 with the small hydrophilic peroxide, EtOOH, produced an initial rapid decrease in the Soret absorbance (data not shown), reflecting the formation of Intermediate I (Scheme I). The observed rate for Intermediate I formation increased linearly with the EtOOH concentration for both PGHS-1 and -2 (Fig. 1A). The slopes of the lines fitted to the data in Fig. 1A were used to estimate $k_1$ values of $3.4 \times 10^6$ M⁻¹ s⁻¹ for PGHS-1 and $0.5 \times 10^6$ M⁻¹ s⁻¹ for PGHS-2. Thus, the initial reaction of PGHS-1 with EtOOH was approximately 7-fold faster than the corresponding reaction of PGHS-2.

Observations of the reactions with EtOOH at 424 nm, which reflect formation of Intermediate II (Scheme I), revealed different patterns for the two isoforms (Fig. 1B). With PGHS-1, the observed rate for Intermediate II formation initially increased with the EtOOH concentration, indicating that step 1 in the mechanism shown in Scheme I was rate-limiting. The observed rate leveled off above 100 μM EtOOH, indicating that step 2 in Scheme I became rate-limiting at higher peroxide levels. The plateau value estimated from fitting the data to a hyperbolic equation, 80 s⁻¹, provides an estimate for the first order rate constant ($k_2$) with PGHS-1. In contrast, with PGHS-2 the observed rate of Intermediate II formation increased linearly as the EtOOH level was raised, without any indication of a plateau, even at observed rates approaching 300 s⁻¹ (Fig. 1B). Thus, the value of $k_2$ for PGHS-2 in reaction with EtOOH must be well above 300 s⁻¹, and conversion of Intermediate I to Intermediate II is clearly much faster for PGHS-2 than for PGHS-1.

Reaction of PGHS-1 and -2 with 15-HPETE—Reaction of PGHS-1 and -2 with the fatty acid hydroperoxide, 15-HPETE, led to rapid formation of Intermediate I, as indicated by the decrease in the Soret absorbance (data not shown). The observed rate for Intermediate I formation increased linearly with 15-HPETE concentration for both PGHS isoforms (Fig. 2A). The second order rate constant ($k_1$) estimated from the data was $2.3 \times 10^7$ M⁻¹ s⁻¹ for PGHS-1 and $2.5 \times 10^7$ M⁻¹ s⁻¹ for PGHS-2, indicating that the two isoforms have very similar reactivity with this lipid hydroperoxide. On the other hand, observations of the kinetics of Intermediate II formation revealed divergent behavior for PGHS-1 and -2. For PGHS-1, the observed rate for Intermediate II formation increased linearly at lower 15-HPETE concentrations but began to level off at peroxide concentrations above 100 μM, indicating that the second step in Scheme I was becoming rate-limiting (Fig. 2B). A plateau was not reached, due to dead time limitations of the stopped flow instrument, but fitting of the data to a hyperbolic equation indicated a $k_2$ value of approximately 900 s⁻¹.
PGHS-1, the observed rate of Intermediate II formation was slower than that for Intermediate I at all 15-HPETE levels. For PGHS-2, the observed rate of Intermediate II formation was indistinguishable from that for Intermediate I formation throughout the 15-HPETE concentration range tested (Fig. 2B, inset), indicating that the first step was always rate-limiting and precluding estimation of a $k_2$ value. However, Intermediate II formation was so much faster for PGHS-2 than for PGHS-1 at all 15-HPETE levels used (Fig. 2B), so the $k_2$ value must be much greater for PGHS-2 than PGHS-1.

**Reaction of PGHS-2 with PPHP—**The peroxidase intermediate kinetics of PGHS-2 were also examined with a second hydrophobic hydroperoxide, PPHP. The observed rate for formation of Intermediate I was essentially the same as that for Intermediate II at each of the PPHP levels tested (Fig. 3). This is the same result found for 15-HPETE (Fig. 2) and again indicates that the first step in Scheme I remained rate-limiting and that the value of $k_2$ for PGHS-2 is quite large with both PPHP and 15-HPETE. The value of $k_1$ for PGHS-2 with PPHP estimated from the data in Fig. 3 is $1 \times 10^7$ M$^{-1}$ s$^{-1}$, quite comparable with the value obtained with 15-HPETE above.

Kinetic scan experiments were carried out for the reaction of PGHS-2 with PPHP to examine the spectral changes in more detail (Fig. 4). The Soret peak was found to simultaneously decrease in intensity and shift to longer wavelengths as the reaction proceeded, with one isosbestic point near 414 nm. This is in marked contrast to the behavior of PGHS-1 where the decrease in Soret intensity, which reflects conversion of resting enzyme to Intermediate I, occurred before the red shift, reflecting conversion of Intermediate I to Intermediate II (7, 19, 23). Further, in PGHS-1 there is an isosbestic point between resting enzyme and Intermediate I near 424 nm in reactions with lipid hydroperoxides (7, 19, 23), quite distinct from the isosbestic point observed at 414 nm for PGHS-2 (Fig. 4). The coordinated diminution and red shift of the Soret band observed during reaction of PGHS-2 with PPHP suggests that resting enzyme is converting to Intermediate II without significant transient accumulation of Intermediate I. This prominence of resting enzyme and Intermediate II as the principal species during reaction of PGHS-2 with PPHP is entirely consistent with the observation that the first step is rate-limiting for both hydrophobic hydroperoxides in the single wavelength stopped flow experiments (Figs. 2 and 3).

**Effects of Intermediate II Formation Rate on Cyclooxygenase Kinetics—**Kinetic simulations were used to predict the effect of changes in the rate of Intermediate II formation on the overall cyclooxygenase kinetics, in particular the requirement of the cyclooxygenase for hydroperoxide activator. Experimentally, the hydroperoxide activator requirements for PGHS-1 and -2 are estimated from the sensitivities of the two cyclooxygenase activities to inhibition by added hydroperoxide scavenger enzyme, GSP (6). In this process, the cyclooxygenase velocity achieved by a fixed amount of PGHS is measured in the presence of increasing amounts of GSP. The ratio of added GSP activity to control cyclooxygenase activity ($R_{GC}$) needed for complete cyclooxygenase suppression is used as an empirical measure of the efficiency of cyclooxygenase activation by hydroperoxide. The value of this end point $R_{GC}$ was found to be about 75 for PGHS-1 and 700 in PGHS-2 (6). Simulations of the
The cyclooxygenase kinetics were carried out by numerical integration of rate equations derived from a mechanistic model (Scheme II) as described under “Materials and Methods.” Experimentally based estimates are available for each of the rate constants in the model except for $k_6$, the rate of tyrosyl radical quenching by reducing cosubstrate.

The sensitivity of the system to the value of $k_6$ was explored using values of the other parameters appropriate for PGHS-1, including a value of 350 s$^{-1}$ for $k_2$. The simulations predicted that the cyclooxygenase activity becomes more easily suppressed by GSP as the $k_6$ value increases, with the end point $R_{GC}$ value decreasing from about 130 for $k_6 = 1000$ M$^{-1}$ s$^{-1}$ to about 7 for $k_6 = 2 \times 10^4$ M$^{-1}$ s$^{-1}$ (Fig. 5). The inverse relationship between the $k_6$ value and the end point $R_{GC}$ is readily apparent from the inset in Fig. 5. A $k_6$ value of 2000 M$^{-1}$ s$^{-1}$ predicted an end point $R_{GC}$ close to the experimentally observed value of 75 for PGHS-1 (6). The sensitivity of the system to the value of $k_2$ then was explored with several values of $k_6$ (Fig. 6). The end point $R_{GC}$ was predicted to increase as the $k_2$ value was increased, with most of the change occurring between $k_2$ values of 350 and 2000 s$^{-1}$. Regardless of the $k_6$ value chosen, at saturating $k_2$ values the end point $R_{GC}$ reached a plateau about 50% over the value predicted for a $k_2$ of 350 s$^{-1}$ (Fig. 6).

**DISCUSSION**

The cyclooxygenase activity of PGHS-1 has long been known to require hydroperoxides for initiation (24). This requirement for a hydroperoxide activator leads to a strong positive feedback loop because the cyclooxygenase product, PGG$_2$, is itself a hydroperoxide (Scheme I). The feedback loop is thought to be comprised of the hydroperoxide-dependent generation of a tyrosyl radical (steps 1 and 2 in Scheme I) and the formation of additional hydroperoxide in cyclooxygenase catalysis itself (step 5 in Scheme I); the overall pattern is that of an autocatalytic branched chain reaction (25). More recently, it has been established that cyclooxygenase activity of PGHS-2 also requires a hydroperoxide activator (5, 6). The observation that the PGHS-2 cyclooxygenase is activated at hydroperoxide levels approximately 10-fold lower than those needed for PGHS-1 (6) indicates that the positive feedback loop is more efficient in PGHS-2 than in PGHS-1. This difference could conceivably originate at any of the steps in the feedback loop. Given the similar cyclooxygenase specific activities observed for the two human isoforms expressed in the same system (26), however, it seems unlikely that the more efficient activation in PGHS-2 is due to more efficient cyclooxygenase propagation (step 5 in Scheme I). Rather, attention focuses on differences in the formation (steps 1 and 2 in Scheme I) or dissipation (step 6 in Scheme I) of the catalytically active tyrosyl radicals on Tyr$^{385}$ in PGHS-1 and on Tyr$^{371}$ in PGHS-2.

Formation of the key tyrosyl radical is proposed to involve oxidized enzyme intermediates in the peroxidase cycle (7). The initial reaction with hydroperoxide at the heme site leads to a two-electron oxidation of resting enzyme to form Intermediate I (step 1 in Scheme I). This species is analogous to Compound I in horseradish peroxidase and carries one oxidizing equivalent on the ferryl iron and the other as a porphyrin free radical (23). The next step in the postulated activation process (step 2 in Scheme I) is an intramolecular electron transfer from a tyrosine residue in the cyclooxygenase site (Tyr$^{385}$ of PGHS-1 and Tyr$^{371}$ of PGHS-2) to the porphyrin, forming a tyrosyl radical and bringing the heme to one oxidizing equivalent above the resting state (7). This intramolecular electron transfer mechanistically links the cyclooxygenase and peroxidase catalytic cycles and distinguishes the PGHS isoforms from other heme-dependent peroxidases that do not have an oxygenase activity (27, 28).

The results presented here show that the rates of formation of Intermediate I during reaction of the two PGHS isoforms with the fatty acid hydroperoxide, 15-HPETE, are essentially the same (Fig. 2). Therefore, it is reasonable to expect similar behavior for PGG$_2$, the relevant fatty acid hydroperoxide formed during cyclooxygenase catalysis with arachidonate. PGHS-1 does have a higher Intermediate I formation rate than PGHS-2 for reaction with EtOOH (Fig. 1), presumably reflecting structural differences between the isoforms at the peroxidase active site that favor interactions of PGHS-1 with small hydrophilic peroxides. The major kinetic difference between the two isoforms found in the present study was in the rate of formation of Intermediate II. The rate was much faster for PGHS-2 than for PGHS-1 with both hydrophilic and hydropho-
bic hydroperoxides (Figs. 1 and 2). For PGHS-1, formation of Intermediate I was rate-limiting at low hydroperoxide levels, whereas conversion of Intermediate I to Intermediate II was rate-limiting at higher hydroperoxide levels. For PGHS-2, interconversion of Intermediate I to II was so fast that formation of Intermediate I was rate-limiting at all hydroperoxide levels tested, and there was no appreciable accumulation of Intermediate I (Fig. 4). Thus, it is clear that the final step in the process of cyclooxygenase activation by lipid hydroperoxide is distinctly faster in PGHS-2 than in PGHS-1.

Although PGHS-1 and -2 share 60% overall amino acid identity, the conservation is much higher in the regions around the peroxidase and cyclooxygenase active sites (2). The three-dimensional structures are also well conserved in the active sites, with differences in backbone positions of the two isoforms averaging less than 0.4 angstrom (8–10). As a result, there are no readily apparent structural differences in the vicinity of the heme and Tyr385 (Tyr371) in the available crystallographic data (8, 10) that explain the observed differences in the value of $k_2$ in PGHS-1 and -2. It remains possible that the active site structures in the crystals differ from those of the active enzymes in solution or that differences in structural dynamics lead to the observed differences in electron transfer rate.

The potential effects of the faster rate of Intermediate II formation in PGHS-2 on overall cyclooxygenase kinetics need to be considered in the context of the cellular environment in which the PGHS isoforms operate. Most cells have a large excess of peroxide scavenging enzymes, such as GST, over peroxide generating enzymes, such as the cyclooxygenases (21). This preponderance of peroxide scavenging capacity tends to keep the cellular hydroperoxide level well below those encountered in vitro and may thereby accentuate the impact of differences in activation efficiency. Indeed, analyzing the effects of added peroxide scavengers has revealed features of feedback activation by hydroperoxide that are not apparent in routine cyclooxygenase assays (22, 29, 30), and titration with GST has been used to quantify the strength of the feedback loops in PGHS-1 and -2 (6). Kinetic modelling of the complex combination of PGHS and GST is thus very useful in predicting how differences in individual rate constants might influence cyclooxygenase catalysis in vivo.

The kinetic behavior of systems containing both PGHS and GST can readily be predicted using numerical integration of equations based on mechanistic models (6, 22), and so we used this approach to predict the effect of changes in the $k_2$ value on the sensitivity of the cyclooxygenase to inhibition by hydroperoxide scavenger. The mechanistic model chosen for kinetic simulations (Scheme II and “Materials and Methods”) is based on the branched chain tyrosyl radical mechanism proposed by Ruf and colleagues (Ref. 7; see also Scheme I). The mechanism was modified to include two additional intermediates (E(III)/PPPIX/Tyr* and E(IV)/PPPIX/Tyr* in Scheme II) to permit redox cycle events at the peroxidase site to continue after generation of the tyrosyl radical in the cyclooxygenase site. A simple route to events at the peroxidase site to continue after generation of the Tyr* and E(IV)/PPIX*/Tyr* in Scheme II) to permit redox cycle modifications (Scheme II and “Materials and Methods”) is based on the sensitivity of the cyclooxygenase to inhibition by hydroperoxide scavenger by up to 50% (Fig. 6). This is less than the 8-fold difference in resistance to inhibition by GST actually observed for the two cyclooxygenase activities (6). It appears that the increased rate of Intermediate II formation ($k_Q$) observed here for PGHS-2 compared with PGHS-1 can account for only part of the difference in hydroperoxide activation efficiency between the two isoforms. The simulation results also indicate that the resistance of the cyclooxygenase activity to inhibition by GST is quite sensitive to the stability of the tyrosyl radical in Intermediate II, with the resistance increasing as the value of the $k_R$ rate constant was decreased (Fig. 5). With a $k_R$ value of 250 M$^{-1}$ s$^{-1}$ and a $k_2$ value of about 2000 s$^{-1}$, the predicted end point $R_G$ value was above 600 (Fig. 6), close to the end point $R_G$ value of 650 actually observed for human PGHS-2 (6). The ability of the mechanistic model to simulate the GST sensitivity of PGHS-2 once the $k_R$ value is decreased suggests that the active site tyrosyl radical in PGHS-2 is less readily quenched by reducing cosubstrates than the corresponding tyrosyl radical in PGHS-1. Electron paramagnetic resonance kinetic measurements will be needed to test this intriguing possibility that differing tyrosyl radical stabilities in the two PGHS isoforms also contribute to the difference in hydroperoxide activator efficiency.

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