Rapid Kinetics of Tyrosyl Radical Formation and Heme Redox State Changes in Prostaglandin H Synthase-1 and -2*

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Hydroperoxide-induced tyrosyl radicals are putative intermediates in cyclooxygenase catalysis by prostaglandin H synthase (PGHS)-1 and -2. Rapid-freeze EPR and stopped-flow were used to characterize tyrosyl radical kinetics in PGHS-1 and -2 reacted with ethyl hydrogen peroxide. In PGHS-1, a wide doublet tyrosyl radical (34–35 G) was formed by 4 ms, followed by transition to a wide singlet (33–34 G); changes in total radical intensity paralleled those of Intermediate II absorption during both formation and decay phases. In PGHS-2, some wide doublet (30 G) was present at early time points, but transition to wide singlet (29 G) was complete by 50 ms. In contrast to PGHS-1, only the formation kinetics of the PGHS-2 tyrosyl radical matched the Intermediate II absorbance kinetics. Indomethacin-treated PGHS-1 and nimesulide-treated PGHS-2 rapidly formed narrow singlet EPR (25–26 G in PGHS-1; 21 G in PGHS-2), and the same line shapes persisted throughout the reactions. Radical intensity paralleled Intermediate II absorbance throughout the indomethacin-treated PGHS-1 reaction. For nimesulide-treated PGHS-2, radical formed in concert with Intermediate II, but later persisted while Intermediate II relaxed. These results substantiate the kinetic competence of a tyrosyl radical as the catalytic intermediate for both PGHS isoforms and also indicate that the heme redox state becomes uncoupled from the tyrosyl radical in PGHS-2.

Prostaglandin H synthase (PGHS) catalyzes the first committed step in the biosynthesis of prostanoids. There are two isoforms of PGHS, with the constitutive form (PGHS-1) ascribed housekeeping functions and the inducible form (PGHS-2) associated with cytokine-mediated pathophysiological events (1, 2). Although the isoforms share only 60% sequence identity, their overall three-dimensional structures are very similar, especially in the catalytic sites (3–5). Both isoforms exhibit two enzymatic activities: a cyclooxygenase activity, which converts arachidonic acid to prostaglandin G2, and a peroxidase activity, which transforms prostaglandin G2 to prostaglandin H2. Several oxidized reaction intermediates have been identified (6–9). As indicated in Scheme 1, PGHS first interacts with a peroxide substrate, such as prostaglandin G2, to generate Intermediate I, equivalent to Compound I of the horseradish peroxidase. Intermediate I then converts to Intermediate II, equivalent to complex ES of cytochrome c peroxidase, through an intramolecular electron transfer from a tyrosine residue to the oxidized porphyrin. The transient tyrosyl radical in Intermediate II in each PGHS isoform is capable of oxidizing arachidonic acid to generate an arachidonic acid radical and initiate cyclooxygenase catalysis (10, 11). Crystallographic data revealed that the presumed site of the tyrosyl radical, Tyr-385 in PGHS-1 (Tyr-371 in PGHS-2), is located between the heme and arachidonate binding sites, well positioned to couple the two enzyme activities (3–5), as proposed in the original branched-chain mechanism (7, 8) (Scheme 1).

Although the chemical competence of the tyrosyl radical for cyclooxygenase catalysis has been convincingly demonstrated by single-turnover experiments (10, 11), the kinetic competence of the tyrosyl radical had been examined only with manual mixing techniques (13). Thus, the relationship between tyrosyl radical kinetics and those of the rapid changes in the heme redox state during PGHS peroxidase reactions remained unresolved. The redox linkage between the heme center and the tyrosyl radical needs to be defined to determine the coupling efficiency between the two enzyme activities.

Several types of tyrosyl radical EPR spectra have been observed in the PGHS isoforms (Refs. 7, 12–16 and Table I). A 33–35-G wide doublet (WD1) and wide singlet (WS1) are observed when PGHS-1 is manually mixed with various hydroperoxides (12). On the other hand, only a 29-G wide singlet (WS2) is found in PGHS-2 for similar manually mixed peroxidase reactions (16, 24). It was of interest to know whether the lack of observable WD in PGHS-2 was the result of a fundamentally different tyrosyl radical structure in this isoform or of a more rapid transition to a wide singlet. The peroxidase reaction in PGHS-1 treated with cyclooxygenase inhibitors such as aspirin, indomethacin, and flurbiprofen or with the mutation of Tyr-385 to phenylalanine resulted in the formation of a 25–26-G narrow singlet (NS1a) (12, 17, 18). A similar 21-G narrow singlet (NS2a) was observed for PGHS-2 treated with the cyclooxygenase inhibitor nimesulide or with the mutation of Tyr-371 (11, 16). These observations of a perturbed tyrosyl radical EPR in PGHS-1 and -2 with impaired cyclooxygenase activity were from manually mixed samples. It remained important to determine whether other radicals were generated earlier in the rapid reactions with peroxide.

We have used rapid-freeze EPR to measure the tyrosyl radical kinetics during reaction of PGHS-1 and -2 with EtOOH,
both with and without pretreatment of the enzymes with cyclooxygenase inhibitors. Parallel stopped-flow measurements were done to determine the kinetics of Intermediate I and II formation. The results indicate that very rapid formation of WD species coincides with formation of Intermediate II in both PGHS-1 and -2, consistent with the proposed mechanism (Ref. 7 and Scheme 1), and the formation of the narrow singlet EPR spectra from reaction of PGHS-2, the large value of Ayz for the beta carbon hydrogens; Ayz = 9.52, Ayz = 27.4, and Ayz = 7.22 G for the 3-, and 5-phenyl hydrogens, with a line width of 5 G.

**EXPERIMENTAL PROCEDURES**

Phenol, indomethacin, flurbiprofen, nimesulide, and hemin were purchased from Sigma. EtOOH was purchased as a 5% aqueous solution. EtOOH concentrations were determined by double integration of the EPR signal using a copper standard (12) and a packing correction factor of 0.45 determined earlier for this system (19). Rapid-freeze experiments were conducted using a System 1000 chemical/freeze quench apparatus with a Model 1019 syringe ram, a Model 715 ram controller, and an 0.008-in nozzle (Update Instrument, Leatherhead, UK). Wavelength pairs of 428 and 524 nm (isosbestic points between resting PGHS-1 and Intermediate I (8, 9)) were used to monitor changes in Intermediate II in PGHS-1. In the case of PGHS-2, the large value of h2 (conversion of Intermediate I to Intermediate II) resulted in a minimal accumulation of Intermediate I, and so there was hardly any observable accumulation of Intermediate I (26). We thus chose to monitor Intermediate II kinetics in PGHS-2 at 406 and 428 nm.

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Rapid-freeze experiments were conducted using a System 1000 chemical/freeze quench apparatus with a Model 1019 syringe ram, a Model 715 ram controller, and an 0.008-in nozzle (Update Instrument, Madison, WI). A low temperature isopentane bath was used for pre-chilling the packing assembly containing isopentane and later for cooling during sample packing via a pressure filtration process developed recently in our laboratory (19). The bath temperature was kept at 125–130 K, bringing the temperature of isopentane inside the funnel to 130–135 K. The temperatures were monitored with a silicon diode temperature sensor (Lakeshore model 200 monitor with a DT-471-PA1 probe). Unless otherwise mentioned, the ram velocity was 2 cm/s in all rapid-mixing experiments. Single-push mixing was used for reactions of less than 160 ms; longer reactions used a push-push mixing syringe program. The dead time of the apparatus, measured with myoglobin oxyhemoglobin reactions, is 4–5 ms (19). EPR spectra of samples collected from rapid-freeze experiments were recorded with a Varian E-6 spectrometer. The EPR conditions were as follows: modulation amplitude, 3.2 G; time constant, 1 s; power 1 mW; and temperature, 96 K. Radical concentrations were determined by double integration of the EPR signal using a copper standard (12) and a packing correction factor of 0.45 determined earlier for this system (19).

**RESULTS**

**Kinetics of PGHS-1 Tyrosyl Radicals**—To observe the earliest possible events in tyrosyl radical formation, we reacted PGHS-1 with two equivalents of EtOOH at 0 °C and freeze-trapped the sample at various reaction times. The sample trapped at 4 ms showed a typical wide-doublet tyrosyl radical (WD1) with a peak-to-trough width of 34 G and a well resolved 19-G center splitting (Fig. 1). A sample trapped after 5 s of reaction still showed a doublet spectrum but with some decay of the center splitting. Comparison of the freeze-trapped samples with the wide-doublet EPR predicted from the parameters obtained in electron nuclear double resonance (ENDOR) meas-

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**TABLE 1**

| Nomenclature | Origin | EPR features |
|--------------|--------|-------------|
| **WD1**      | PGHS-1 | 34–35 G, 19 G splitting |
| **WD2**      | PGHS-2 | 30 G |
| **WS1**      | PGHS-1 | 33–35 G |
| **WS2**      | PGHS-2 | 29 G |
| **NS1a**     | PGHS-1 | 25–26 G, hyperfine structure |
| **NS1b**     | PGHS-1 | 25–28 G, no hyperfine features |
| **NS2a**     | PGHS-2 | 21 G |

**SCHEME 1. Branched-chain radical mechanism of PGHS.** AA, arachidonic acid; PGGL, prostaglandin G2; PGHL, prostaglandin H2; ROOH and ROH, hydroperoxide and corresponding alcohol.
increased by 30% at 4 ms (Fig. 1). Increasing the ratio of EtOOH/PGHS-1 to 5 and raising the temperature permitted observation of a more complete transition from WD1 to a wide singlet tyrosyl radical (WS1) (Fig. 2, top). This transition was essentially complete within 50 s. The initial formation of tyrosyl radical was rapid ($k_{16}$ s$^{-1}$) and reached a plateau at 0.6 spin/heme within 300 ms (Fig. 2, bottom). A slow decay in radical intensity ($k_{0.047}$ s$^{-1}$) began after about 500 ms (Fig. 2, bottom). The kinetics of Intermediate II, monitored at $A_{524}$, closely tracked those of the tyrosyl radical, indicating that binding of indomethacin did not disrupt the redox linkage between the heme and the tyrosyl radical. Separate experiments indicated that the center splitting in the WD1 EPR had already declined by $-30\%$ at 4 ms (Fig. 1).

Effects of Cyclooxygenase Inhibitors on PGHS-1 Tyrosyl Radical Kinetics—The EPR spectra of samples trapped at various times during reaction of indomethacin-treated PGHS-1 with EtOOH (5 eq) all showed a 25–26-G narrow singlet (NS1a) with discrete hyperfine features (Fig. 3, top) just as was found earlier in manual mixing experiments (12, 17). There was no indication of initial formation of either WD1 or WS1 signals. Formation of the NS1a radical was rapid ($k = 47$ s$^{-1}$) and reached a plateau at $-0.35$ spin/heme within 200 ms, followed by a slow relaxation (Fig. 3, bottom). As in the case of PGHS-1 itself, the kinetics of Intermediate II (29 s$^{-1}$ for the initial phase) paralleled those of the NS1a radical, indicating that binding of indomethacin did not disrupt the redox linkage between the heme and the tyrosyl radical. Separate experi-

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ments using flurbiprofen instead of indomethacin gave very similar results for both the tyrosyl radical and Intermediate II kinetics (data not shown).

**Kinetics of PGHS-2 Tyrosyl Radicals**—Substantial formation of tyrosyl radicals was evident at the earliest stage (5 ms) of reaction of PGHS-2 with 5 eq of EtOOH (Fig. 4, top). The EPR spectrum of the initial sample exhibited a complex, 29–30-G wide signal with a clearly discernible splitting, whereas the spectrum of the sample at 50 ms was essentially a simple singlet (WS2), indicating a very rapid transition from doublet to singlet in PGHS-2. Subtraction of 75% of the signal intensity of the 50-ms sample from the 5-ms sample EPR yielded a doublet with a peak-to-trough width of 30 G (Fig. 4, top), indicating the presence of a substantial wide doublet tyrosyl radical in the very early stages of the PGHS-2 peroxidase reaction.

The formation of tyrosyl radical in PGHS-2 was rapid (−140 s⁻¹). The radical intensity reached a plateau near 0.8 spin/heme by 50 ms and remained at this level for at least 100 ms (Fig. 4). Formation of Intermediate II, monitored by absorbance changes at 406 or 428 nm, was also rapid (−240 s⁻¹). In contrast to the persistent radical intensity, Intermediate II quickly relaxed back to the ferric heme (17–19 s⁻¹). This difference in behavior between the heme center redox state and the tyrosyl radical was observed for PGHS-2 but not PGHS-1 (compare Figs. 2 and 4).

The PGHS-2 preparation used above included 50 μM phenol as a protectant, and therefore the possible effect of phenol on the PGHS-1 tyrosyl radical and Intermediate II kinetics was examined. In the presence of phenol the rates of tyrosyl radical and Intermediate II formation (18 and 26 s⁻¹, respectively) were similar to those for phenol-free PGHS-1 reactions (Figs. 2 and 5). The maximal tyrosyl radical intensity in the presence of phenol was −0.4 spin/heme, somewhat less than in the experiment done without phenol (Figs. 2 and 5). The presence of phenol resulted in a slower transition from WD1 to WS1, evident from the center inflection present in the EPR even after 330 ms (Fig. 5, top). This finding contrasts with the almost complete collapse of the center splitting pattern in the phenol-free PGHS-1 reactions (Fig. 2, top). A comparison of the PGHS-1 and -2 results in the presence of phenol indicated that the transition from wide doublet to wide singlet and the relaxation of Intermediate II were both slower in PGHS-1 (Figs. 4 and 5).

**Tyrosyl Radical Kinetics in Nimesulide-treated PGHS-2**—Reaction of nimesulide-treated PGHS-2 with 5 eq of EtOOH resulted in rapid formation of both the tyrosyl radical (176 s⁻¹) and Intermediate II (−350 s⁻¹ from Δ428 nm observations) (Fig. 6). Intermediate II later decayed at a rate of 33 s⁻¹ even though the tyrosyl radical intensity remained relatively constant (Fig. 6, bottom). The divergent decay kinetics of oxidized heme and tyrosyl radical were thus seen with both inhibitor-treated and native PGHS-2 (Figs. 4 and 6). The maximal intensity of the tyrosyl radical achieved in nimesulide-treated PGHS-2, −0.4 spin/heme, was substantially lower than that found for PGHS-2 itself. EPR of samples collected throughout the reaction showed only the 21-G narrow singlet (NS2a), with no significant change in line shape (Fig. 6). There was no sign of

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**Fig. 4.** **Radical and ferryl heme kinetics during reaction of PGHS-2 with EtOOH.** PGHS-2 (11.2 μM) in 0.1 M potassium phosphate, pH 7.2, containing 0.1% Tween 20 and 10% glycerol was reacted with 56 μM EtOOH at 24 °C. **Top panel,** EPR spectra were acquired for samples freeze-trapped at 5 (solid line) and 50 ms (dashed line) and normalized to the same amplitude. The dotted line is the difference spectrum obtained by subtracting 75% of the 50-ms spectrum from the 5-ms spectrum. **Bottom panel,** the kinetics of the tyrosyl radical (solid circles and short-dashed line for a single-exponential fit) were obtained by double integration of the EPR spectra. The heme kinetics were monitored in parallel stopped-flow experiments monitored at 406 (continuous line) and 428 nm (long dashes).
earlier formation of either a wide doublet or wide singlet, a situation similar to that with indomethacin-treated PGHS-1 (Figs. 3 and 6).

**DISCUSSION**

The rapid formation of WD and WS tyrosyl radicals observed in both PGHS-1 and -2 parallels the formation of the oxyferryl heme species (Intermediate II). This finding is just as predicted by the branched-chain radical mechanism originally proposed by Ruf and colleagues (7, 8). The kinetic competence of the tyrosyl radical for the proposed coupling of the peroxidase and cyclooxygenase activities in both of the PGHS isoforms is thus firmly established.

We previously examined the time-dependent changes of tyrosyl radical concentration and EPR line shape on a time scale of seconds using manual mixing at lower temperatures (-10 to 0 °C) (12, 13, 17). The slower kinetics observed in these earlier experiments probably reflects a previously unsuspected inefficiency in mixing the enzyme and peroxide in the narrow EPR tube by agitation with a nichrome wire loop. Earlier results from single-turnover experiments firmly established the chemical competence of the tyrosyl radical to oxidize arachidonic acid to a fatty acid radical (10, 11, 22, 23). It is clear from the present kinetic results that tyrosyl radicals in both of the PGHS isoforms are formed very rapidly, in concert with heme redox changes, just as expected from the proposed mechanism (Scheme 1). This result provides strong support for the mechanism and indicates that it accurately describes the events leading from the reaction with peroxide to the initiation of cyclooxygenase catalysis in both PGHS-1 and -2.

The present results also make it clear that a wide doublet tyrosyl radical (WD2) species is formed early in the reaction of peroxide with PGHS-2 just as with PGHS-1. The initial wide doublet undergoes a transition to a wide singlet in both isozymes, with a much faster rate for the transition in PGHS-2. Our ENDOR studies support earlier reports (14, 15) that the WS1 of PGHS-1 is not a distinct species but is rather a mixture of the WD1 and a narrow-singlet (NS1b) associated with self-inactivated enzyme (13). This singlet is roughly similar to that seen with PGHS inhibited by anti-cyclooxygenase agents.
similar difference was observed with the NS radicals in PGHS-1 but diverge markedly in PGHS-2 (Figs. 2 and 4). A tyrosyl radical and the heme center parallel one another inmediate I to II (26). Surprisingly, the redox relaxation of the showed PGHS-2 to have a much faster conversion from Intermediate to 2 than for PGHS-1. The faster rate of Intermediate transfer in Compound I, resulting in oxidation of a surrogate tyrosine residue is not known for either of the porphyrin radical, as we previously proposed (18). The identity (Tyr-371 in PGHS-2) during electron transfer to the heme sustains propagation of cyclooxygenase activity. This difference in redox coupling between oxyferryl heme and the tyrosyl radical in the two isozymes may contribute to the greater efficiency of hydroperoxide activation in PGHS-2 than in PGHS-1 (21).

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