Structural Characterization of Arachidonyl Radicals Formed by Prostaglandin H Synthase-2 and Prostaglandin H Synthase-1 Reconstituted with Mangano Protoporphyrin IX*

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A tyrosyl radical generated in the peroxidase cycle of prostaglandin H synthase-1 (PGHS-1) can serve as the initial oxidant for arachidonic acid (AA) in the cyclooxygenase reaction. Peroxides also induce radical formation in prostaglandin H synthase-2 (PGHS-2) and in PGHS-1 reconstituted with mangano protoporphyrin IX (MnPGHS-1), but the EPR spectra of these radicals are distinct from the initial tyrosyl radical in PGHS-1. We have examined the ability of the radicals in PGHS-2 and MnPGHS-1 to oxidize AA, using single-turnover EPR studies. One wide singlet tyrosyl radical with an overall EPR line width of 29–31 gauss (G) was generated by reaction of PGHS-2 with ethyl hydroperoxide. Anaerobic addition of AA to PGHS-2 immediately after formation of this radical led to its disappearance and emergence of an AA radical (AA⁺) with a 7-line EPR, substantiated by experiments using octadeuterated AA. Subsequent addition of oxygen resulted in regeneration of the tyrosyl radical. In contrast, the peroxide-generated radical (a 21G narrow singlet) in a Y371F PGHS-2 mutant lacking cyclooxygenase activity failed to react with AA. The peroxide-generated radical in MnPGHS-1 exhibited a line width of 36–38G, but was also able to convert AA to an AA⁺ with an EPR spectrum similar to that found with PGHS-2. These results indicate that the peroxide-generated radicals in PGHS-2 and MnPGHS-1 can each serve as immediate oxidants of AA to form the same carbon-centered fatty acid radical that subsequently reacts with oxygen to form a hydroperoxide. The EPR data for the AA-derived radical formed by PGHS-2 and MnPGHS-1 could be accounted for by a planar pentadienyl radical with two strongly interacting β-protons at C10 of AA. These results support a functional role for peroxide-generated radicals in cyclooxygenase catalysis by both PGHS isoforms and provide important structural characterization of the carbon-centered AA⁺.

*This work was supported by National Institutes of Health Grants GM44911, GM52170, and GM21337 and the Welch Foundation Grant i. 5,6,8,9,11,12,14,15-octadeuterated arachidonate; WS2, wide-singlet radical found in PGHS-2; MnPGHS-1, MnPGHS-1 reconstituted with mangano protoporphyrin IX; HPETE, hydroperoxyeicosatetraenoic acid.
We have therefore tested the ability of these radicals to oxidize arachidonate to a fatty acid radical, a key step in the branched-chain mechanism in Scheme 1. The results indicate that very similar arachidonoyl free radicals are formed when the fatty acid is reacted with the PGHS-2 and MnPGHS-1 protein radicals. Analysis of the EPR spectrum of the arachidonoyl radicals provides insight into the structure of the enzyme-bound fatty acid radicals in both PGHS isoforms.

MATERIALS AND METHODS

AA was purchased from NuChek Preps, Inc., Elysian, MN; 5,6,8,9,11,12,14,15-octadecanate 1 was purchased from NuChek Preps, Inc., Elysian, MN; and 15-HPETE was synthesized according to Graff et al. (18). Ethyl hydroperoxide (EtOOH) was the product of Polyscience Inc., Warrington, PA, and 15-HPETE was synthesized as described previously (16). Cyclooxygenase activity was calculated from the oxygen consumption rate (19). The batches of PGHS-1 used in this study had specific activities of 55–80 pmol of O_2/min/mg of protein; those of PGHS-2 had specific activities of 27–42 pmol of O_2/min/mg. The Y371F mutant of PGHS-2 was expressed in a baculovirus system and purified as the apoenzyme (20). The Y371F mutant of PGHS-2 was prepared by the same heme reconstitution procedure using procedures developed for wild-type PGHS-2 (21). The Y371F mutant of PGHS-2 was prepared by the same heme reconstitution procedure using procedures developed for wild-type PGHS-2 (21). The Y371F mutant of PGHS-2 was expressed in a baculovirus system and purified as the apoenzyme (20). The Y371F mutant of PGHS-2 was expressed in a baculovirus system and purified as the apoenzyme (20).

RESULTS

Serial Reaction of PGHS-2 with Hydroperoxide and Arachidonate—When PGHS-2 was reacted anaerobically with 1.5 eq of EtOOH, a 29 G wide singlet (denoted WS2) amounting to about 0.1 spin/heme was routinely detected by EPR (Fig. 1, spectrum a). Similar yields of radical have been found under these conditions with several batches of PGHS-2; the yield increased to about 0.3 spin/heme with 5 eq of EtOOH (data not shown). Subsequent anaerobic addition of 1.5 eq of AA led to the replacement of the WS2 radical with a new radical exhibiting a 7-line EPR centered at \( g = 2.0022 \), with a hyperfine splitting of 13.5–14 G and a slightly lower intensity (Fig. 1, spectrum b). Another sample freeze-trapped a minute later exhibited a very similar EPR line shape but with reduced amplitude (Fig. 1, spectrum c). These three samples subsequently were thawed and mixed with air. The EPR intensity of the WS2 signal in PGHS-2 reacted only with EtOOH decreased during aerobic incubation (Fig. 1, spectrum a’). The two samples of PGHS-2 reacted with EtOOH and AA both reformed the original WS2 tyrosyl radical and recovered significant intensity. This regeneration of the hydroperoxide-dependent tyrosyl radical upon aerobic incubation indicates that the radical formed after AA addition itself reacted with oxygen to form fresh hydroperoxide, as is expected from the mechanism (Scheme 1), where the arachidonate radical reacts with oxygen to form the hydroperoxide, PGG_2, and regenerate the tyrosyl radical (Steps 4 and 5).

The 7-line EPR observed for the putative AA in the PGHS-2 reaction (Fig. 1) is distinct from the isotropic AA found earlier in PGHS-1 (11). To determine if this 7-line radical in the PGHS-2 reaction is actually derived from the fatty acid, the single-turnover experiments were repeated with \( d_8 \)-AA. As shown in Fig. 2, spectra b and c, the radical formed upon addition of \( d_8 \)-AA displayed an EPR centered at the same \( g \) value (2.0022) and had similar hyperfine splitting (13.5–14 G) as the radical formed with the unlabeled AA. The main difference is that the radical formed with \( d_8 \)-AA has five hyperfine lines in the EPR spectrum instead of the seven lines observed with unlabeled AA. A 5-line EPR pattern was also observed with PGHS-1 when \( d_8 \)-AA was used as reactant (11). Conversion from a 7-line to a 5-line EPR spectrum with the deuterated fatty acid strongly supports the conclusion that these radicals are derived from the arachidonate substrate. Thawing and aerobic incubation of the samples with deuterated fatty acid radical regenerated the WS2 tyrosyl radical (Fig. 2, spectra b’ and c’), consistent with formation of hydroperoxide by reaction of the \( d_8 \)-AA fatty acid radical with oxygen.

Serial Reaction of Y371F PGHS-2 with EtOOH and AA—
15-HPETE (Fig. 4, 35 G wide doublet was generated upon reaction with 1.5 eq of

Tyr-385 has been shown to be the likely location of the wide doublet tyrosyl radical in PGHS-1 (24). The effects of mutating the corresponding residue in PGHS-2, Tyr-371, to phenylalanine were examined. The cyclooxygenase activity of the Y371F mutant was undetectable, whereas the peroxidase activity was 40–60 μmol of H₂O₂/min/mg, in the range observed for the wild-type activity (34–77 mmol of H₂O₂/min/mg). Thus, the Y371F mutation in PGHS-2 led to selective loss of cyclooxygenase activity, as was observed with the Y385F mutant of PGHS-1 (25).

When Y371F PGHS-2 was reacted with EtOOH, a 21 G radical signal was observed (Fig. 3, spectrum a). This signal is very similar to those found with indomethacin-treated PGHS-1, the Y385F mutant of PGHS-1 (24), and nimesulide-treated PGHS-2 (13), all of which have reduced or undetectable cyclooxygenase activity. Anaerobic addition of AA to the peroxide-induced radical in the Y371F mutant did not cause any EPR spectral line shape change (Fig. 3, spectrum b). Longer incubation with AA led only to the decay of the 21 G signal (Fig. 3, spectrum c). Subsequent thawing and aerobic incubation of the samples did not lead to any changes of the EPR line shape (Fig. 3, spectra a’–c’). These results showed that although mutation of Tyr-371 in PGHS-2 did not prevent radical formation, the resulting radical was not able to oxidize AA.

Serial Reaction of MnPGHS-1 with 15-HPETE and AA—A 35 G wide doublet was generated upon reaction with 1.5 eq of 15-HPETE (Fig. 4, spectrum a). Subsequent anaerobic addition of 1.5 eq of AA converted this wide doublet to an isotropic radical with about the same line width, centered at g = 2.003 (Fig. 4, spectrum b). A sample freeze-trapped after another 30 s of reaction with AA (spectrum c in Fig. 4) displayed the same EPR spectral line shape but only 50% of the intensity of that in spectrum b. These three samples were subsequently thawed and incubated aerobically. Aerobic incubation had little effect on the intensity or line shapes of MnPGHS-1 incubated with 15-HPETE alone (spectrum a’). For the MnPGHS-1 samples incubated with 15-HPETE and AA, aerobic incubation led either to subtle changes of EPR line shape (spectrum b’) or a significant increase in amplitude (spectrum c’).

To see if the isotropic MnPGHS-1 signals in spectra b and c in Fig. 4 are derived from arachidonic acid, these experiments were repeated with d₈-AA in place of AA. The resulting spectra are shown in Fig. 5. The EPR signal from enzyme reacted with 15-HPETE and d₈-AA exhibited five hyperfine lines with splitings of 13–14 gauss and centered at g = 2.003 (spectra b and c, respectively). Thawing and oxygenation of the samples reacted with 15-HPETE and d₈-AA restored the 34–35 G radical signal found in the initial reaction of MnPGHS-1 with peroxide (Fig. 5, spectra b’ and c’). This change in EPR line shape, from an isotropic signal with unlabeled AA to a 5-line pattern with d₈-AA, confirmed that the radicals in spectra b and c in Figs. 4 and 5 were derived from the fatty acid substrate, similar to the previous observations with FePGHS-1 (11). In all cases, the radical concentrations peaked at about 0.1 spin/metalloporphyr-
rin, as was previously observed for reaction between 15-HPETE and MnPGHS-1 (16).

Formation of Carbon-centered Arachidonate Radical under Aerobic Conditions—In a separate experiment, concentrated FePGHS-1 (150 μM heme) in air-saturated buffer was reacted with 25 eq of AA. A radical with a 7-line spectrum centered at $g = 2.002$ was obtained with hyperfine splitting of 12–16 G and a concentration of about 0.11 spin/heme (Fig. 6, top). The EPR lineshape of this radical species is very similar to that of the AA carbon-centered radical generated by PGHS-2 (Fig. 1, spectra b and c). Under these experimental conditions, the dissolved oxygen initially present in the FePGHS-1 reaction mixture
with conversion of the AA original tyrosyl radical(s) as predicted by Scheme 1, consistent taglandin formation is blocked by the lack of oxygen during the in FePGHS-1, MnPGHS-1, and FePGHS-2. In this study, pros- tor between the metalloporphyrin center and the AA substrate competent to play a common functional role as a redox media-
that a peroxide-generated, protein-linked radical is chemically oxidize AA to form an AA produced radicals in PGHS-2 and MnPGHS-1 are both able to results of the present study demonstrate that the peroxide-in-
substited singlet (33–35 G) tyrosyl radical generated in FePGHS-1 by peroxide can oxidize AA and produce a carbon-centered AA uncter (Table I) with a line width of 4.3 G (top spectrum) or 5 G (bottom spectrum).

(--300 μM) would be enough to sustain only one catalytic cycle (2 mol of O₂/mol of PGG₂) for the concentrated enzyme used. With very limited oxygen replenishment by diffusion into the unstirred solution in the narrow EPR tube, anaerobiosis was achieved in situ, stopping the cyclooxygenase cycle at the stage of the fatty acid radical (Scheme 1) in this sample.

DISCUSSION

Oxidation of AA by Peroxide-induced PGHS Radicals—Oxi-
dation of arachidonate to a fatty acid radical is a key step in the proposed branched-chain mechanism for PGHS cyclooxygenase catalysis (Step 3 in Scheme 1). It has been demonstrated previously that the wide doublet (33–35 G) tyrosyl radical generated in FePGHS-1 by peroxide can oxidize AA and produce a carbon-centered AA radical. The tyrosyl radical induced by peroxide in PGHS-2 is a wide singlet (29–30 G), quite distinct from the initial wide doublet tyrosyl radical in FePGHS-1 (11–13); the peroxide-induced radical in MnPGHS-1 also has an EPR spectrum distinct from the radical in FePGHS-1 (16). The results of the present study demonstrate that the peroxide-induced radicals in PGHS-2 and MnPGHS-1 are both able to oxidize AA to form an AA radical (Figs. 1, 2, 4, and 5). This establishes that a peroxide-generated, protein-linked radical is chemically competent to play a common functional role as a redox mediator between the metalloporphyrin center and the AA substrate in FePGHS-1, MnPGHS-1, and FePGHS-2. In this study, prostaglandin formation is blocked by the lack of oxygen during the anaerobic portion of the reactions. Subsequent incubation of the AA radical with air for several seconds leads to regeneration of the original tyrosyl radical(s) as predicted by Scheme 1, consistent with conversion of the AA radical to PGG₂. Formation of prostaglan-
dins during aerobic reaction of FePGHS-1 and MnPGHS-1 with arachidonate on a similar time scale has already been demonstrated (16, 27).

Several lines of evidence have been previously cited (13) to suggest that the wide singlet radical observed in PGHS-2 upon reaction with EtOOH resides on Tyr-371 (corresponding to Tyr-385 in FePGHS-1). Assignment of the PGHS-2 radical to Tyr-371 and the involvement of this residue in cyclooxygenase catalysis are strengthened by the present results with the Y371F mutant of PGHS-2. This mutant lacked cyclooxygenase activity, demonstrating the requirement for a tyrosine residue at position 371 of PGHS-2. The Y371F mutant retained peroxidase catalytic competence, as expected from the proposed mechanism (Scheme 1). Although the Y371F mutant generated a narrow singlet radical when reacted with peroxide, this radical did not oxidize AA (Fig. 3). The 21G narrow singlet radical in the PGHS-2 Y371F mutant has a line shape very similar to those in nimesulide-treated PGHS-2 or PGHS-1 inhibited by aspirin or indomethacin, each of which has impaired cyclooxygenase activity and normal peroxidase activity (13, 28). The narrow singlet in inhibitor-treated PGHS-1 has been proposed to reside on a tyrosine residue other than Tyr385 (24). The similarities with the present results suggest that the 21G narrow singlet found in the PGHS-2 Y371F mutant is a tyrosyl radical formed on a residue other than Tyr-371. The narrow singlet tyrosyl radical induced in indomethacin-treated PGHS-1 by EtOOH was not able to oxidize AA (11), but this could be ascribed to blockage of the cyclooxygenase site by indomethacin. The Y371F PGHS-2 mutant radical is also unable to oxidize AA (Fig. 3), but in this case, no inhibitor is present in the cyclooxygenase channel. This establishes clearly that AA is not oxidized by the Y371F mutant radical even though the fatty acid can freely access its binding site, making it likely that the radical in the Y371F mutant is some distance from the fatty acid site or not properly positioned to interact with the fatty acid. The residue bearing the radical has yet to be identified.

MnPGHS-1 has been found to resemble FePGHS-1 in overall peroxidase mechanism and formation of high oxidation-state intermediates (29). In MnPGHS-1, the radical is generated more efficiently by lipid hydroperoxides than by EtOOH, as might be expected from the preference of the MnPGHS-1 peroxidase activity for lipid substrates (16). Accumulation of the radical correlates roughly with cyclooxygenase catalysis by MnPGHS-1 (16), just as for FePGHS-1 (27). For both MnPGHS-1 and FePGHS-1, reaction with the tyrosine nitrating agent, tetrynitromethane, leads to formation of a peroxide-induced radical with a narrower EPR spectrum and loss of cyclooxygenase activity (16, 26). The present results extend the parallels between MnPGHS-1 and FePGHS-1, showing that the MnPGHS-1 radical is also able to oxidize arachidonate to a fatty acid radical (Figs. 4 and 5). This implies that the peroxide-
generated radical in MnPGHS-1 is near the fatty acid binding site. A simple interpretation is that the wide doublet radical species in both MnPGHS-1 and FePGHS-1 are tyrosyl radicals residing on Tyr-385. If this is the case, the EPR differences between the MnPGHS-1 and FePGHS-1 radicals must arise from the influence of the different metal centers, situated about 10 Å away from Tyr-385 in the crystal structure (8). The effect of the metal on the reactivity of the radical toward the fatty acid remains to be evaluated.

Structure of the Arachidonic Acid Carbon-centered Radical—

Dramatic changes of EPR line shape, from an isotropic singlet for MnPGHS to a 7-line hyperfine pattern (when d8-AA was the substrate), in Fig. 7). This is because the latter would give, at most, a 4-line allyl radical (delocalized on three carbons, C11-C13 or C13-C15 if the carbon-centered radical is most likely a pentadienyl radical (31, 32). Acceptable simulations of the observed AA radicals observed in reactions with unlabeled and deuterated arachidonate (Table I and Figs. 1, 4, 6, and 7). The optimal values for the A tensor constants used in the simulation. For example, the change of the A tensor value measured for the endo proton of a model pentadienyl radical (9.6 G) to that of an isolated radical found for a methyl proton in the ethyl radical model (23 G), can be used to estimate a ρ value of 0.42 (33). Taken with the PGHS-2 and MnPGHS-1 EPR data (Table I), two sets of β proton dihedral angles can be calculated: 32° and 152° for the carbon atom where both protons interact strongly with the adjacent unpaired electron, and 51° and −71° for the carbon with only one strongly interacting proton. To decide which set of dihedral angles is associated with β protons on C10 and which is associated with those on C16, we considered the geometric conformation of the AA: in the cyclooxygenase site and its subsequent chemical conversion to PGG2, involving endoperoxide formation across C9 and C11 and ring closure between C8 and C12. We favor assignment of the 32°/152° pair to C10 and the 51°/−71° pair to C16 because this arrangement places C9 closer to C11 for endoperoxide formation and also brings C8 closer to C12 for subsequent ring closure (Fig. 7). These assignments fix the structure from C9-C17 of the carbon-centered AA: formed in the first step of cyclooxygenase reaction in PGHS-2 and MnPGHS-1.

The values of the parameters which produced optimal EPR simulations for the AA: EPR of PGHS-2 and MnPGHS-1 are very similar (Table I). The main difference is a 2 G smaller coupling constant for the C13 proton for the MnPGHS-1 EPR. The EPR line shape is a sensitive function of the coupling constants used in the simulation. For example, the change of (H1/H3) from 9.5 to 11.5 G and of line width from 5 to 4 G resulted in a dramatic change of the predicted EPR appearance from a broad isotropic singlet for MnPGHS to a 7-line hyperfine pattern for PGHS-2 (compare Fig. 1, spectrum b and Fig. 4, spectrum b). Obviously, the experimental resolution of hyperfine features is dependent on many factors such as the inherent line width, temperature, sample homogeneity, etc. Using d5-AA significantly enhanced the resolution as the line broadening effects of protons at C11 and C15 were removed from the spectra, leading to a consistent 5-line hyperfine EPR spectrum originating from interactions of the unpaired electron and only the four protons at C10, C13, and C16.

In our earlier provisional analysis (11), a 5-proton system was used to simulate the FePGHS-1 AA: EPR and a 3-proton system for the d5-AA: EPR, assuming that only one β-proton each on C10 and C16 was interacting strongly with the unpaired electron at C11 and C15. These earlier simulations also did not attempt to use a consistent set of A tensor values for AA: and d5-AA: and thus provided limited information about the fatty acid radical structure. The current EPR spectra for the AA: generated by PGHS-2, MnPGHS-1, and concentrated FePGHS-1 at high AA are clearly better interpreted as originating from a pentadienyl radical with not one but two strongly interacting β-protons at C10 (Figs. 1, 2, 4–6). We therefore
have reanalyzed the earlier PGHS-1 AA EPR spectra (11) in light of the new data. However, the narrow line width of the PGHS-1 AA EPR, 23 G, did not permit a satisfactory fit to the EPR data for both AA and d₆-AA radicals with the individual basic parameter sets tested. Possible explanations for this difference in EPR lineshape are discussed below.

It is important to note that the radical formed under oxygen-limiting conditions at high concentrations of enzyme and arachidonate exhibited a typical 7-line EPR very similar to that found for the AA in PGHS-2 (Figs. 1 and 6). This 7-line EPR found for concentrated PGHS-1 is easily simulated using the same parameter values used to simulate the PGHS-2 AA, with only a slight increase in line width (4.3 G instead of 4 G; Table I). Interestingly, a similar radical EPR spectrum was obtained earlier by Lassmann et al. (17) for concentrated MnPGHS-1 reacted with a high concentration of AA (Fig. 6). This 7-line EPR with MnPGHS-1 is also simulated easily by the same set of parameters used for PGHS-2, with a line width of 5 G (Table I). These results help to resolve one of the remaining puzzles about the identity of the radical generated using high enzyme and substrate concentrations: why was an EPR similar to that found for the AA observed instead of a tyrosyl radical? We propose that the oxygen dissolved in the reaction mixture was exhausted in a very few turnovers when high concentrations of enzyme and fatty acid are present, thus trapping the cyclooxygenase reaction at the carbon-centered AA stage, as predicted by the mechanism in Scheme 1. This conclusion that further turnover of the enzyme was blocked by oxygen exhaustion is supported by the observation that only 1 eq of total eicosanoid products were formed by MnPGHS-1 (17). As mentioned above, the EPR line shape of the AA generated in FePGHS-1 at high concentrations of enzyme and substrate (Fig. 6) does differ from the singlet observed earlier at low enzyme and substrate concentration (11). This could be due to different AA structures under the two sets of reaction conditions. However, the simulated EPR line shape of the AA is highly sensitive to the effects of small changes in coupling constants (Table I and Figs. 1b and 4b). We thus suggest that several aspects of the different appearances of AA spectra probably result from slight differences in factors that determine the inherent EPR line width of a common planar pentadienyl radical. It does remain possible that differences in the kinetics of AA formation with high and low enzyme concentrations may lead to trapping of AA with different conformations. In any case, the AA structure may not be the same as that of enzyme-bound AA, because substantial conformational changes accompany pentadienyl radical formation, and adaptive changes in the protein could occur to stabilize the carbon-centered radical.

In summary, the present results show that the peroxide-induced tyrosyl radical in FePGHS-2 and the peroxide-induced radical in MnPGHS-1, which is probably also a tyrosyl radical, are both capable of oxidizing arachidonate to a carbon centered fatty acid radical. This provides strong support for a key step in the tyrosyl radical mechanism proposed for PGHS cyclooxygenase catalysis. Interpretation of the EPR spectra provides a detailed description of the structures of the arachidonyl radicals formed in FePGHS-1 and -2 and in MnPGHS-1.

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