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Structural comparisons of arachidonic acid-induced radicals formed by prostaglandin H synthase-1 and -2

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Abstract

Cyclooxygenase catalysis by prostaglandin H synthase (PGHS)-1 and -2 involves reaction of a peroxide-induced Tyr385 radical with arachidonic acid (AA) to form an AA radical that reacts with O₂. The potential for isomeric AA radicals and formation of an alternate tyrosyl radical at Tyr504 complicate analysis of radical intermediates. We compared the EPR spectra of PGHS-1 and -2 reacted with peroxide and AA or specifically deuterated AA in anaerobic, single-turnover experiments. With peroxide-treated PGHS-2, the carbon-centered radical observed after AA addition was consistently a pentadienyl radical; a variable wide-singlet (WS) contribution from mixture of Tyr385 and Tyr504 radicals was also present. Analogous reactions with PGHS-1 produced EPR signals consistent with varying proportions of pentadienyl and tyrosyl radicals, and two additional EPR signals. One, insensitive to oxygen exposure, is the narrow singlet tyrosyl radical with clear hyperfine features found previously in inhibitor-pretreated PGHS-1. The second type of EPR signal is a narrow singlet lacking detailed hyperfine features that disappeared upon oxygen exposure. This signal was previously ascribed to an allyl radical, but high field EPR analysis indicated that ~90% of the signal originates from a novel tyrosyl radical, with a small contribution from a carbon-centered species. The radical kinetics could be resolved by global analysis of EPR spectra of samples trapped at various times during anaerobic reaction of PGHS-1 with a mixture of peroxide and AA. The improved understanding of the dynamics of AA and tyrosyl radicals in PGHS-1 and -2 will be useful for elucidating details of the cyclooxygenase mechanism, particularly the H-transfer between tyrosyl radical and AA.

Keywords

PGHS radical; EPR; High-field EPR; Radical dynamics

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2010.11.012.

1. Introduction

The two prostaglandin H synthase (PGHS) isozymes catalyze the first committed step of prostanoid biosynthesis. Although the two isozymes of PGHS play very different roles in physiology and pathology [1,2], they have quite similar structures: the cyclooxygenase active site residues of the two isozymes are almost superimposable, with a root mean square deviation of less than 0.4 Å [3–5].

Both isozymes have two catalytic activities: a cyclooxygenase that transforms AA to PGG₂, and a peroxidase that converts PGG₂ to PGH₂ [6,7]. A branched-chain radical mechanism that couples the two catalytic activities [8,9] has gained solid support from crystallographic and spectroscopic data [3–5,10,11]. The critical oxidant for cyclooxygenase catalysis in this mechanistic model is a peroxide-induced intermediate containing a tyrosyl radical. Mutagenic and crystallographic data identified Tyr385 (in ovine PGHS-1 numbering) as the location of the catalytic radical [3–5,10–12].

The EPR spectra of peroxide-treated PGHS-1 and -2 change over time, with wide-doublet signals (WD1 and WD2 for PGHS-1 and -2, respectively) predominating initially and wide-singlet signals (WS1 and WS2 for PGHS-1 and -2, respectively) appearing later [13–15]. The transition from WD to WS is much faster in PGHS-2 than in PGHS-1 [16]. These WS signals are made up of the original WD and a narrow singlet spectrum (NS1 and NS2 for PGHS-1 and -2, respectively) [14]. Results from EPR studies of peroxide generated radicals in PGHS-1 and -2 with mutated tyrosine residues indicate that the WD→WS spectral transition involves formation of tyrosyl radicals at Tyr385 and Tyr504, with the Tyr385 radical (WD) dominating in the early stages and the Tyr504 (NS) predominating later [17–19]. When PGHS is first complexed with some cyclooxygenase inhibitors, reaction with peroxide generates narrow singlet tyrosyl radicals, NS1a for PGHS-1 and NS2 for PGHS-2 [13–15,20]. NS1a is similar to NS1b (generated when PGHS-1 is reacted with >20 fold excess AA aerobically) in EPR lineshape but NS1a has better-defined hyperfine features [14,21,22].

EPR observations in single turnover experiments convincingly demonstrated that peroxide-generated tyrosyl radical in PGHS-1 and -2 oxidizes arachidonic acid (AA) to generate an AA-derived carbon-centered pentadienyl radical [23,24] that later reacts with oxygen and cyclizes to form PGG₂. Studies using PGHS-2 and specifically-deuterated AAs provided firm support for the formation of a pentadienyl radical delocalized over C11–C15 of the substrate [25,26]. However, these results did not address the structure of the substrate derived radical(s) in PGHS-1, which has a considerably smaller, and potentially more constraining, cyclooxygenase active site [3–5]. We report here the results of a comparative structural study on the AA-induced radical(s) of the two PGHS isoforms using the single-turnover EPR approach. We found five different overall EPR signatures in reactions of peroxide-activated PGHS-1 with AA, each representing either a single species or a mixture of radicals, whereas only two EPR signatures were observed with PGHS-2. Surprisingly two of the five EPR signatures observed with PGHS-1 originate from a tyrosyl radical, one identical to the tyrosyl radical observed for inhibitor-treated PGHS-1 (NS1a) [14], and the other a novel species (NS1c). Moreover, a time-dependent EPR transition (WD1/WS1→pentadienyl radical→NS1c/NS1a) was observed with PGHS-1 in a rapid-freeze quench experiment, with the intermediate spectra capable of being resolved by global analysis. These results will facilitate kinetic analyses of early events in cyclooxygenase catalysis by PGHS-1 and -2.

2. Materials and methods

AA was purchased from NuChek Preps, Inc., Elysian, MN. 5,6,8,9,11,12,14,15-octadeuterated arachidonic acid (d_8 -AA) was a gift from Hoffman-La Roche, Nutley, NJ., or purchased from Cayman Chemical, Ann Arbor, MI. Characterization of d_8 -AA has been described previously [23,24]. 10,10- d_2 -AA, 11- d -AA, 13(R)- d -AA, 15- d -AA, 13(R),15- d_2 -AA and 16,16- d_2 -AA were prepared in high stereochemical and isotopic purity by total synthesis using a common precursor, 11-*al*-undeca-5,8-dienoic acid methyl ester [25–27]. Hemin was obtained from Sigma. Ethyl hydroperoxide (EtOOH) was a product of Polyscience Inc., Warrington, PA, or a gift from Dr. G. Barney Ellison, University of Colorado; procedures for determining EtOOH concentration and purity have been described previously [28].

Ovine PGHS-1 was purified from sheep seminal vesicles [29] and human PGHS-2 was purified from recombinant material expressed in a baculovirus system [30]. The holoenzymes were prepared by replenishing with heme; excess heme was removed by passage over DEAE-cellulose [31]. Purified PGHS-2 requires phenol (~50 μ M) to protect against activity loss during reconstitution [16], whereas PGHS-1 appears to have sufficient endogenous reductant [32]. Cyclooxygenase activity was determined from the oxygen consumption rate [29]; specific activities were 100–120 μ mol O_2 /min/mg for PGHS-1 and 20–40 μ mol O_2 /min/mg for PGHS-2.

Single-turnover experiments using an anaerobic titrator were performed at 0 °C as previously described [23,24]. Rapid-freeze quench EPR experiments followed our published procedure [16] but without oxygen present. Anaerobic solutions of PGHS-1 and the EtOOH/AA mixture were prepared in a tonometer on an anaerobic train, and the rapid-freeze apparatus (System 1000, Update Instrument, Madison, WI), was positioned inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). All glassware, mixers and reaction tubings were transferred to the anaerobic chamber one day prior to the experiment. The oxygen level in the chamber was monitored by an oxygen/hydrogen analyzer (Model 10, Coy Laboratory Products) and remained <5 ppm (<0.01 μ M in solution) during the entire experiment. Single or double push programs were used to obtain samples freeze-trapped at different reaction times. For D-band sample preparation, the quartz tube was connected directly to the nozzle outlet; the tube was removed after filling with reaction mixture, wiped quickly, and immersed in liquid nitrogen to freeze within 5 s. Samples prepared in the X- or D-band tubes are stable in liquid nitrogen storage for several months without obvious changes in their EPR.

X-band EPR was recorded at liquid nitrogen temperatures on a Varian E-6 or Bruker EMX spectrometer [23]. The routine EPR conditions were: modulation amplitude, 2.0 or 3.2 G; time constant, 1 s; power, 1 mW; and temperature, 96 K (Varian) or 110 K (Bruker). Radical concentrations were determined by double integration of the EPR signals, with reference to a copper standard (15). Most computer simulations of EPR spectra used a modification of the POWFUN program [33] kindly provided by Drs. Gerald T. Babcock and Curt Hoganson, Michigan State University. Data analysis and simulation for spectra obtained with 10,10- d_2 -AA and 16,16- d_2 -AA were carried out using the WIN-EPR and SimFonia software provided with the Bruker EMX system. The accuracy of spectral simulations was evaluated by normalizing to a spin concentration of 1 and calculating the standard error of estimate (S.E.E.):

$$\text{S.E.E.} = \left\{ \sum (y'_i - y_i)^2 / (n - 1) \right\}^{\frac{1}{2}} / \left\{ \sum y_i'^2 / (n - 1) \right\}^{\frac{1}{2}} \quad (1)$$

where y_i' is the EPR signal amplitude, y_i is the simulated amplitude at the same magnetic field and n is the number of data points. A smaller S.E.E. value indicates a more accurate simulation.

Global analyses of EPR spectra obtained in the rapid freeze quench kinetic experiments in regard to putative reaction mechanisms were conducted using the Pro-K program (Applied Photophysics, Leatherhead, UK) by importing ASCII data for the EPR acquired at different time points. Pro-K is a multi-variable regression analysis based on Marquardt–Levenberg algorithm to obtain the optimized values for nonlinear (rates) and linear (amplitude) parameters in a mechanistic model [34].

HFEPR spectra were collected on the D-band (130 GHz) spectrometer at Albert Einstein College of Medicine, as previously described [35,36]. The spectrometer has a 7 T Magnex superconducting magnet with a 0.5 T sweep/active shielding coil. Field swept spectra were recorded in the two-pulse, echo-detected mode with the following parameters: temperature, 7 K; repetition rate, 10 Hz; 90 ° pulse, 40 ns; and time between pulses, 130 ns. The magnetic field was calibrated to an accuracy of ~3 G using a sample of manganese-doped MgO [37]. The temperature of the sample was maintained within ± 0.3 K using an Oxford Spectrostat cryostat and an ITC503 temperature controller. The D-band spectra were simulated as described previously [38,39], with hyperfine interactions treated to first order and transition probabilities taken as unity. The distribution in g_x was modeled as a Gaussian: spectra with the appropriate g -values were calculated, weighted according to their position in the distribution, and summed to generate the final simulation.

3. Results

3.1. EPR signals produced during anaerobic reaction of peroxide-treated PGHS-1 and -2 with AA

Reaction of PGHS-1 or -2 with 2–10 eq of EtOOH efficiently produced mixtures of Tyr385 and Tyr504 radicals that equilibrated within ~20 s at 0 °C and gave rise to characteristic wide-singlet tyrosyl radical EPR signals (WS1 and WS2 for PGHS-1 and -2, respectively) [14]. The WS1 and WS2 tyrosyl radical mixtures maintained EPR lineshape and intensity long enough that they could be reacted manually with AA under anaerobic conditions for EPR analysis of the early steps in cyclooxygenase catalysis. The initial task was to distinguish EPR signals contributed by AA radical intermediates from those due to tyrosyl radicals, a task made easier by the use of specifically deuterated AA substrates. Representative examples of the types of EPR spectra obtained from >150 such manual single-turnover experiments with unlabeled and deuterated AA for the two PGHS isozymes are shown in the Supporting Information (Figs. S1–S6). The interpretation of each type of spectrum in terms of the contributing radicals is summarized in Scheme 1. The experiments with PGHS-2 produced overall EPR spectra consistent with a pentadienyl AA radical alone or a mixture of pentadienyl AA and the WS2 tyrosyl radicals (Fig. S1). The results with PGHS-1 were more variable and complex. In addition to the pentadienyl AA radical alone and mixtures of the pentadienyl and WS1 tyrosyl radicals, three other EPR signatures were observed in some of the single-turnover experiments. One was a narrow singlet tyrosyl radical similar to the NS1a observed when inhibitor-pretreated PGHS-1 was reacted with peroxide (Fig. S3). The second additional type of EPR spectrum observed was similar to that attributed previously to an allyl AA radical [40]. Further analysis by HFEPR (see later text) indicated that the latter spectrum was actually a novel tyrosyl radical signal that will be referred to as NS1c. Although NS1c has an X-band EPR very similar to NS1a, NS1c forms only in the presence of AA. The final type of EPR signal observed in single-turnover experiments with PGHS-1 was a mixture of NS1c tyrosyl radical and pentadienyl AA radical (Fig. S6). No systematic correlation was apparent between the particular type of EPR

spectrum observed in a particular experiment with the cyclooxygenase specific activity, the EtOOH stoichiometry (1–10 eq), or the AA stoichiometry (1–5 eq). In at least one case, duplicate experiments with a single batch of PGHS-1 resulted in different types of EPR spectrum. The two EPR patterns for PGHS-2 occurred with almost equal frequency. For PGHS-1, the NS1c or pentadienyl AA radical+NS1c types of spectrum occurred in >70% of the experiments.

We used both ‘dissection’ and ‘synthesis’ approaches for resolution of EPR patterns presumed to be composed of two spectral components. In the ‘dissection’ analysis, a variable proportion of an authentic EPR signal of one component (“A”) observed in a separate experiment was subtracted from the observed composite EPR spectrum to generate a series of putative spectra that were evaluated as candidates for a pure second component (“B”) (See Figs. S5 and S6 in Supporting Information). In the ‘synthesis’ approach, we first calculated the theoretical EPR spectra for equal spin concentrations of the putative A and B components using published spectroscopic parameters [24,41]. Variable proportions of the two calculated spectra were combined to generate a series of composite simulated EPR spectra that were evaluated for their fit to the observed EPR spectrum (e.g., Fig. S5 in Supporting Information).

3.2. Time-dependent changes of tyrosyl and substrate radicals in PGHS-1

One possible explanation for the variability in EPR line shape observed in the manual single-turnover experiments with peroxide-treated PGHS-1 and AA was that it reflected variability in the efficiency of mixing and trapping, thus producing samples at different stages of reaction. We thus conducted rapid-freeze EPR kinetic measurements using PGHS-1. It was shown previously that the sequence of EtOOH and AA addition had little influence on the type of radical intermediates freeze-trapped [23], so we decided to use one-stage mixing to simplify the process and conserve enzyme. Anaerobic reactions of PGHS-1 with a 1:1 mixture of EtOOH and AA were sampled at various times from 6 ms to 10 s and analyzed by EPR (Fig. 1). Formation of a mixture of WD1 and WS1 tyrosyl radicals reached a maximum as early as 20–50 ms (Fig. 1, 20 ms spectrum). At 100–500 ms the EPR appeared to be a composite of a pentadienyl AA radical signal and either WD1 or WS1 components (Fig. 1, 500 ms spectrum), as indicated by comparison with arithmetically generated spectra for mixtures of the individual species (Fig. S5, right panel). The presence of pentadienyl radical was supported by the trough at ~3350 G and the low-field side band of the main peak, signals that persisted for at least 2 s (arrows in Fig. 1, 2 s spectrum). The main EPR changes between 0.5 and 2 s were the substantial decrease in overall line width and the transformation to a less symmetric shape, with the trough much wider than the peak. The spectral lineshape at 2 s was very similar to the composite spectrum generated by the summation of spectra from a pentadienyl radical and either an NS1a or NS1c tyrosyl radical (data not shown). The NS1b tyrosyl radical, observed under strongly inactivating conditions [14], seemed unlikely to be a major component here because the present samples retained substantial cyclooxygenase activity (Lü et al., unpublished observation). Reaction samples trapped after 4 s showed only a ~24 G narrow EPR without the satellite shoulders (Fig. 1, 4.5 s spectrum). This narrow EPR appearing at long reaction times did not have the clear hyperfine features observed for the NS1a tyrosyl radical and was probably the NS1c tyrosyl radical. Unfortunately, the presence of isopentane precluded thawing the rapid-freeze samples to test the narrow species for regeneration of the WD1/WS1 signature after exposure to oxygen.

3.3. Global analysis of EPR kinetic data for species forming during reaction of PGHS-1 with EtOOH and AA

We first validated the global analysis procedure with EPR spectra from fourteen time points (10 ms–50 s) during the simpler reaction of PGHS-1 with EtOOH alone, where the WD1 signal formed, converted to WS1, and subsequently decayed (Fig. 2A). Using the mechanism shown in Fig. 2B, the global analysis was able to resolve the WD1 and WS1 line shapes (Fig. 2C) and accurately reconstructed the experimental spectra (Fig. 2D). Similar results were obtained with EPR spectra from twelve time points in a second reaction of PGHS-1 with EtOOH (data not shown). The rate constants for the mechanism in Fig. 2B obtained from exhaustive global analysis of spectra from these two reactions were: $k_1=(2.5\text{--}4.0) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_2=0.8\text{--}1.1 \text{ s}^{-1}$; and $k_3=(2.5\text{--}9.0) \times 10^{-3} \text{ s}^{-1}$. The formation rate of WD1 was comparable to the published rate for Intermediate II formation [16]. This ability to extract realistic spectra for the intermediate species and to closely replicate the experimental spectra throughout the course of the reaction indicated that the deconvolution process worked well with real data obtained in an RFQ-EPR experiment.

Initial attempts at global analysis of RFQ-EPR spectra from reactions of PGHS-1 with EtOOH and AA (Fig. 3A), based on the two-branch mechanism (mechanism **a** in Fig. 3B) were unable to fully resolve a pentadienyl radical spectrum (data not shown). The discrepancies, particularly in the center region of the spectra, suggested that an additional, narrower species was present and that the two-branch mechanism was inadequate.

In the second round of global analysis for the PGHS-1/EtOOH/AA EPR data, the mechanism was modified by adding another branch to the mechanism (mechanism **b** in Fig. 3B) and the NS1c signal was specified for the narrow species. As before, AA binding was assumed to be faster than the H transfer between AA and tyrosyl radicals, and thus not rate limiting. These changes allowed accurate deconvolution of the WD1 and pentadienyl radical spectra (Fig. 3C), and realistic reconstruction of experimental EPR spectra obtained during the reaction (Fig. 3D). The optimized k_1 of $2.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ obtained by global analysis fell within the range of $(2.5\text{--}4.0) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ obtained above for reaction of PGHS-1 and EtOOH alone. The optimal rates of other steps were: $k_2=0.5 \text{ s}^{-1}$; $k_3=0.1 \text{ s}^{-1}$; $k_4=0.7 \text{ s}^{-1}$; $k_5=0.1 \text{ s}^{-1}$ and $k_6=0.002 \text{ s}^{-1}$. The analysis predicted accumulation of significant pentadienyl radical between 0.5 and 5 s, peaking at ~30% of total spin at 2 s. No distinction was made between NS1c and NS1a in the global analysis due to the subtle EPR line shape differences between them. In any case, the global analysis provided an effective method to obtain a relatively simple reaction mechanism that can account for the RFQ-EPR kinetic data with both peroxide and AA present.

3.4. Characterization of substrate radical structure in reactions of PGHS-1 and -2 with 10,10-d₂-AA

The contributions of most of the key protons of the AA pentadienyl radical to the hyperfine EPR features have been assessed by previous single-turnover EPR experiments using various deuterated AA substrates [27]. We have now examined the contributions of the two C10 protons using sequential addition of EtOOH and 10,10-d₂-AA to PGHS-1 and -2. With both enzymes, reaction mixtures freeze-trapped ~30 s after 10,10-d₂-AA addition had EPR spectra with a poorly-resolved 5-line hyperfine structure (Fig. 4, top two spectra). Deuterium replacement at C10 thus resulted in dramatic loss of resolution and decrease in overall linewidth, indicating that both C10 hydrogens were strongly coupled to the pentadienyl radical. As expected, a parallel PGHS-2 control experiment using d₈-AA gave a well-resolved 5-line EPR (Fig. 4, bottom spectrum). Formation of substrate radical was quite efficient in all cases, reaching ~0.3 spin/heme. Quite reasonable simulations of the spectra in Fig. 4 were obtained using the same set of isotropic hyperfine coupling constants published

earlier for the pentadienyl radical [24,27], with 1/7 the coupling constants and $I=1$ for the deuterated positions (red dashed lines in Fig. 4). The simulations of the 10,10-d₂-AA radical spectra visually appeared somewhat inferior to that for the d₈-AA radical, but the S.E.E. values were comparable (0.21, 0.20 and 0.22 for PGHS-1 with 10,10-d₂-AA, and PGHS-2 with 10,10-d₂-AA and d₈-AA, respectively). Overall, our data indicated that there were indeed two strongly coupled beta protons at C10 of the pentadienyl radical, as proposed earlier [24]. Loss of the two strongly coupled protons by deuterium replacement resulted in a dramatic loss of resolution and decrease in overall linewidth.

3.5. D-band EPR analysis of radical intermediates in PGHS-1/AA complex reacted with EtOOH

D-band (130 GHz) EPR (HFEPR) has increased sensitivity to the g -value, facilitating the resolution and identification of multiple species. Fig. 5 shows the D-band spectrum of PGHS-1 equilibrated anaerobically with AA first and then reacted with EtOOH. X-band spectrum of this sample was dominated by the species of narrow EPR linewidth (data not shown) attributed to an “allyl” radical in earlier studies [40]. Surprisingly, the HFEPR spectrum of the PGHS-1/AA/EtOOH reaction mixture (Fig. 5, spectrum A) had g -values in the range common for tyrosyl radicals, not fatty acid carbon-centered radicals. Attempts were made to simulate the observed spectrum using parameters consistent with a pure tyrosyl radical (Fig. 5, spectrum B), but the fit was unsatisfactory in the high field (low g -value) spectral region. This region ($g=2.002$ – 2.004) would be expected to contain contributions from π -type radicals with the spin localized primarily on carbon and without substantial spin delocalization on “heavy” atoms such as oxygen or sulfur. The AA pentadienyl radical species fits this description. Accordingly, a 10% contribution of a simulated AA pentadienyl radical signal (Fig. 5, spectrum C) was combined with a 90% contribution from the simulated tyrosyl radical spectrum in Fig. 5B, and the composite spectrum was found to have an acceptable fit to the observed HFEPR spectrum for the PGHS-1/AA/EtOOH sample (Fig. 5, spectrum A). The small contribution of the AA radical species and the lack of resolved hyperfine couplings precluded unambiguous estimates for the hyperfine coupling constants for the AA radical, although its g -values must be in the range of 2.002–2.004. Thus, reaction of the AA complex of PGHS-1 with EtOOH under anaerobic conditions produced a 9:1 mixture of tyrosyl radical (NS1c) and a carbon-centered radical, which may be a pentadienyl radical. The existence of such low concentration of pentadienyl radical was obviously not detectable in X-band EPR spectra.

The simulation of the tyrosyl radical signal in the HFEPR spectrum of the PGHS-1/AA/EtOOH sample (Fig. 5A) required a distribution in the value of g_x (the highest g -value), as had been observed in other tyrosyl radical spectra, including WD [35]. This distribution was attributed to site-to-site differences in the environment of the tyrosyl oxygen atom, such as a distribution in hydrogen bond lengths [42]. This explanation had been confirmed for the WD radical species using HFEPR and HFENDOR [43]. It is thus reasonable to assign the g_x distribution in the NS1c spectrum to a distribution in H-bond lengths of the parent tyrosyl radical.

The NS1c tyrosyl radical was clearly different from WD1, as evident from differences in both X- and D-band spectra [35,43]. The linewidths of the X-band spectra of NS1c and NS1a differed slightly but the latter shows more defined hyperfine features. The D-band spectra of NS1c further revealed a smaller g_x value, 2.0070 vs. 2.0078, but with much larger distribution than that for NS1a radical (Fig. 5 and Ref. [35]). It was not possible to properly simulate NS1c HFEPR data as a composite of NS1a plus a carbon-centered radical. The requirement for AA in NS1c formation also distinguishes it from NS1a with respect to the chemical origin. Thus the radical species giving rise to the NS1c EPR spectrum is probably

different from that responsible for NS1a. However, independent methods will be needed to identify the location of NS1c in the protein.

4. Discussion

4.1. Substrate-derived radicals in PGHS-2

EPR characterization of substrate-derived radicals in anaerobic, single-turnover reactions of PGHS-2 with several specifically-deuterated AAs (Fig. 4 and Refs. [24,26]) has solidly confirmed the formation of a carbon-centered pentadienyl AA radical upon reaction of AA with the peroxide-induced Tyr385 radical. Monodeuterated AA labeled at the 11-, 13(R)-, and 15-positions, dideuterated AAs such as 13(R),15-d₂-, 16,16-d₂- and 10,10-d₂-AA, and 5,6,8,9,11,12,14,15-octadeuterated AA (d₈-AA) were also used in these manual freeze-trapping EPR studies. A pentadienyl radical formed between C11 and C15, with one strongly-coupled proton at C16 and two strongly-coupled protons at C10, satisfactorily explains the observed EPR data [24–26] (Fig. 4). Conversion of tyrosyl radical to the AA radical was sometimes incomplete, as observed in this study (Figs. S4–S6 and Fig. 1). However, in PGHS-2 the interpretation was relatively straightforward as the observed signals were consistent with either a pure pentadienyl radical or a mixture of pentadienyl radical signal and WS2 signal that can be resolved arithmetically (Figs. S4, S5 and S6a). It should be noted that small proportions of WS signal may not alter the lineshape of the pentadienyl radical sufficiently to be detected. The variable presence of residual tyrosyl radicals along with the substrate radical may reflect differences in substrate binding or H-abstraction kinetics. Recent steady-state oxygen kinetic isotope effect measurements with PGHS-1 have indicated that the H-abstraction step is reversible [44], and reaction conditions may affect the kinetics of the forward and reverse reactions in PGHS-2.

4.2. Substrate-derived radicals in PGHS-1

Reactions of PGHS-1 with peroxide and AA produced a more complex variety of EPR spectra than observed with PGHS-2. Five different EPR patterns were observed in the PGHS-1 experiments. Two of these: full conversion from the WD1 or WS1 tyrosyl radical to a pentadienyl substrate radical (Figs. S1 and S3), and partial conversion of the tyrosyl radical to the pentadienyl radical (Fig. S4), are very similar to the observations with PGHS-2. The other three outcomes encountered in the PGHS-1/EtOOH/AA experiments were more complicated and are discussed later.

4.2.1. NS1a alone—In several experiments, only an NS signal, without detectable pentadienyl radical signal was seen in the EPR following addition of AA to EtOOH-treated PGHS-1 (Fig. S3). This NS was not altered when oxygen was introduced. The EPR lineshape of this NS radical was very similar to that of the NS1a species observed when indomethacin-treated PGHS-1 was reacted with hydroperoxide [20]. NS1a appears to arise from the formation of radical at Tyr504 instead of Tyr385 [18,19]. The mechanism by which AA binding would alter the distribution of tyrosyl radical from Tyr385 to Tyr504 is unclear. In contrast to the normal productive binding of AA (Fig. 6 A), the fatty acid substrate has been observed in a reversed binding orientation in PGHS-2 (Fig. 6B and Ref. [45]) and a similar non-productive orientation of AA may be possible in PGHS-1. AA bound in a reverse orientation could plausibly behave similarly to an inhibitor and lead to peroxide-induced radical formation at Tyr504 rather than Tyr385. However, reversed binding should produce marked substrate inhibition, which we have never observed. Furthermore, non-productive AA binding would likely be expected to be reversible, yet the observed NS1a spectrum in the PGHS-1/EtOOH/AA sample was stable, even upon exposure to air (Fig. S3).

4.2.2. NS1c alone—The other NS spectral intermediate found in PGHS-1/EtOOH/AA reactions (Fig. S2) was initially assigned as a sterically strained allyl radical, with constraints imposed by the protein postulated to limit the delocalization of the unpaired electron and the number of strongly interacting protons [40]. The 11d- and 15d-AAAs were designed to determine if the putative allyl radical formed at C11–C13 or at C13–C15. Unfortunately, both of these labeled AAAs led to formation of a radical EPR that is a composite of pentadienyl radical and an NS1c/NS1a tyrosyl radical (Fig. S6). These results provided little useful information about the radical structure other than suggesting it is not substrate based, prompting further structural examination by HFEPR. These HFEPR data (Fig. 5) clearly indicated that the dominant species in the spectrum was a tyrosyl radical, termed NS1c, with a small contribution from a carbon-based radical, likely an AA pentadienyl radical.

Oxygen replenishment to samples whose EPR reflected a mixture of NS1c and pentadienyl radicals caused only partial recovery of the WS1 EPR linewidth. This may reflect conversion of the minor pentadienyl radical component to PGG₂, regenerating the mixture of WD1 and NS1 radicals, or a sequential conversion of part of the NS1c first to a pentadienyl radical and then to a mixture of WD1 and NS1.

4.2.3. Mixtures of pentadienyl radical with NS1a or NS1c tyrosyl radicals—The EPR spectrum observed in some PGHS-1/EtOOH/AA experiments was consistent with a mixture of pentadienyl radical and either NS1a or NS1c (e.g. Fig. S6B). Spectral synthesis using the pentadienyl radical EPR and that of the WS1 tyrosyl radical spectrum failed to reproduce the observed EPR shown in Fig. S6B (data not shown). Whether this NS species was directly converted from the WD1/WS1 tyrosyl radical via phenyl ring rotation or was due to a proton-coupled electron transfer from nearby surrogate tyrosine to Tyr385 is not clear.

The NS1c tyrosyl radical EPR was very different from the WD1 signal, and exhibited subtle differences at X-band from the NS1a signal we observed for inhibitor-treated oPGHS-1 during peroxide reaction [14]. Although all three tyrosyl radicals had g_x parameters indicative of H-bonding, the HFEPR lineshape of WD1 was very different in the g_y and g_z regions. The HFEPR of NS1a has a narrowly-distributed g_x parameter which is not consistent with the HFEPR data in Fig. 5. The X-band EPR spectrum was better simulated as a combination of a pentadienyl radical signal and NS1c than treated as the sum of a pentadienyl radical signal and NS1a (Fig. S6B). We also observed a temperature-dependent EPR lineshape transformation from that of a pentadienyl radical to that of an NS1c in PGHS-1; this interconversion apparently is reversible with a low energy barrier because higher temperature (25 °C vs. 0 °C) favored the NS1c species (Lü et al., accompanying paper). It is important to note that none of the above three EPR outcomes is observed for PGHS-1 reacted with peroxide alone. Instead, a WD1 to WS1 transition is the only observed event in the same reaction time period [20,22–24]. Thus, the NS1a, NS1c and pentadienyl radical components result directly or indirectly from AA binding to, or reacting with, peroxide-treated PGHS-1. Contributions from NS components are not obvious in EPR spectra recorded for anaerobic reactions of PGHS-2 with peroxide and AA [14,40]. It is possible that PGHS-2 may also exhibit NS EPR components in EtOOH/AA reactions under different experimental conditions.

4.3. Structural characterization of radical intermediates in early PGHS reaction steps

The present EPR and HFEPR characterizations of the various radical intermediates in anaerobic reactions of PGHS with peroxide and AA have resolved the long-standing puzzle of why experiments with deuterated and ¹³C-labeled substrates failed to establish the

structure of the putative “allyl” radical. Our data indicates that the EPR spectrum attributed to an allyl radical is actually a combination of a tyrosyl radical, NS1c, and pentadienyl radical signals. The NS1c predominates when the reaction of PGHS-1/AA complex with EtOOH is freeze-trapped for EPR analysis (Fig. 5). On the other hand, nitric oxide (NO)-trapping experiments described in the accompanying paper suggest that pentadienyl radical is the predominant species upon incubation of PGHS-1 with EtOOH and AA (Lü, et al. accompanying paper). These results suggest that an equilibrium is present between AA pentadienyl and NS1c radicals and that NO, presumably acting as an O₂ analog, shifts the equilibrium towards AA radical. The exact location of NS1c is not clear at this moment, but the NS1c tyrosyl radical could serve as the temporary site for the oxidizing equivalent. In the presence of oxygen, formation of the AA peroxy radical would be expected to pull the reaction toward cyclooxygenase catalysis. However, if NS1c really equilibrates with the AA pentadienyl radical, adding air to a sample dominated by NS1c should deplete NS1c and regenerate WD1. This seems in consistent with the actual data (Fig. S2) which showed an increase of total spin but only limited increase of line width. One interpretation for this outcome is that the Tyr385 radical is regenerated upon oxygen replenishment, but experiences a structural conversion to a conformation that produces an NS-type signal rather than the original WD1 signal. Support for this interpretation is provided by the observation that, following the formation of NS1c, PGHS-1 is not inactive but retains the same peroxidase and cyclooxygenase activity as the non-cycled enzyme (Lü et al., accompanying paper).

4.4. Resolution of overlapping EPR contributions of individual radical intermediates

The arithmetic spectral “dissection” and “synthesis” approaches enabled resolution of the radical component EPR in our single-turnover manual freezing EPR experiments (Figs. S1–S6). Global analysis of the kinetic data obtained from rapid-freeze quench EPR also provided the EPR of intermediate spectral components and estimates of the rate constants for individual reaction steps. However, this analysis is highly dependent on the mechanistic model chosen. We sought a “minimal model” that is able to account for the observed kinetic data (Figs. 2 and 3), accepting the possibility that the resolved intermediate species and the associated rates may not be unique if the actual mechanism is more complex. Recent oxygen isotope effect studies suggest that the hydrogen atom-transfer step between the tyrosyl radical and AA is reversible [44]. Allowing reversibility of the hydrogen atom-abstraction step simply adds another variable in the mechanistic model for global analysis and should increase the goodness of fit as the constraints become less rigid. In addition, testing a more complex model requires more time points with finer time increments.

Global analysis of the spectra obtained in rapid-freeze EPR experiments with PGHS-1 was able to resolve the transition of the initial WD1 tyrosyl radical first to a mixture of WD1, NS1 and pentadienyl radicals, then to a mixture of NS1 and pentadienyl radical, and finally to an NS1 radical that could be either an NS1a or an NS1c tyrosyl radical. Global analysis of the EPR spectral kinetics used in this study is a new approach that may be useful in mechanistic studies of other enzymes involving multiple radical intermediates. With the complicated radical dynamics observed for PGHS-1, global data analysis appears to be essential for isolating the key H-abstraction step and provides a useful tool to evaluate kinetic isotope effects with deuterated AA. A full-scale study of kinetic isotope effects on the reaction of PGHS-1 is presented in another paper (Wu et al., accompanying paper).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

PGHS-1 and PGHS-2	prostaglandin H synthase isoform-1 and isoform-2
AA	arachidonic acid
EtOOH	ethyl hydroperoxide
PGG₂	prostaglandin G ₂
PGH₂	prostaglandin H ₂
11-d-AA	11-deuterated AA
13(R)-d-AA	13(R)-deuterated AA
15-d-AA	15-deuterated AA
13(R),15-d₂-AA	13(R),15-dideuterated AA
10,10-d₂-AA	10,10-dideuterated AA
16,16-d₂-AA	16,16-dideuterated AA
d₈-AA	5,6,8,9,11,12,14,15-octadeuterated AA
WD	wide-doublet tyrosyl radical
WS	wide-singlet radical
NS	narrow-singlet tyrosyl radical
NS1a	NS generated by hydroperoxide in indomethacin or flurbiprofen-treated PGHS-1
NS1b	NS generated under highly inactivated conditions
EPR	electron paramagnetic resonance
ENDOR	electron nuclear double resonance
HFEPR	high-field EPR
S.E.E.	standard error of estimate

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Fig. 1.

EPR spectra observed during anaerobic reaction of PGHS-1 with peroxide and AA. PGHS-1 (0.1mM) in 0.1 M KPi, pH 7.3, containing 15% glycerol and 0.1% Tween-20 was mixed at 23 °C with an equal volume of 0.1 MTris, pH 8.0 containing EtOOH (100 μ M), AA (100 μ M) and 0.05% Tween-20. Samples were freeze-trapped after the indicated times at 117–123 K and the EPR spectra were recorded. The spectrometer conditions were: microwave frequency, 9.29 GHz; power, 1 mW; modulation, 2 G; time constant, 0.33 s and temperature, 114–118 K. The vertical lines indicate the peak-to-trough line width for the 20ms sample, and the arrows indicate EPR features associated with the pentadienyl AA radical. Overall

radical intensities (in spin/heme) are shown on the right side of each spectrum. This experiment is one of three repetitions and all of them show similar results.

Fig. 2. Global analysis of EPR spectra obtained during reaction of PGHS-1 (17 μM) with EtOOH (85 μM). A) Experimental EPR spectra for samples quenched at the indicated reaction times; B) kinetic scheme used for global analysis; C) resolved spectra of WD1 and WS1 obtained from global analysis (—) and the corresponding simulated spectra (.....); and D) simulated spectra for the time points in Panel A based on the resolved WD1 and WS1 spectra and their predicted concentrations.

Fig. 3.

Global analysis of EPR spectra obtained during the reaction of PGHS-1 (100 μ M) with EtOOH (100 μ M) and 13, 13- d_2 -AA (100 μ M). A) Experimental EPR spectra for samples quenched at the indicated reaction times; B) kinetic schemes used for global analysis; C) resolved spectra of WD1 and pentadienyl radical obtained from global analysis (—) and the corresponding simulated spectra (·····); and D) simulated spectra for the time points in Panel A based on the resolved spectra and their predicted concentrations. The optimized rate constants for mechanism (b) were: $k_1=2.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_2=0.5 \text{ s}^{-1}$; $k_3=0.1 \text{ s}^{-1}$; $k_4=0.7 \text{ s}^{-1}$; $k_5=0.1 \text{ s}^{-1}$ and $k_6=0.002 \text{ s}^{-1}$.

Fig. 4.

EPR spectra of radicals generated in sequential reactions at 4 °C of PGHS-1 and -2 with EtOOH and 10,10- d_2 -AA. Top: PGHS-1 (15 μ M), was reacted with 3 eq of EtOOH for 21 s before mixing with 2 eq of 10,10- d_2 -AA and freeze-trapping 11 s later. Center: PGHS-2 (10 μ M) was reacted with 10 eq of EtOOH for 21 s before mixing with 4 eq of 10,10- d_2 -AA and freeze-trapping 11 s later. Bottom: 5-line AA radical EPR spectrum generated using EtOOH and d_8 -AA with the same PGHS-2 preparation under similar conditions. Radical intensities (in spin/heme) are shown at right. Simulated spectra (dashed red lines) for all three samples used the same parameters for the pentadienyl AA radical except that the spectra generated for the 10,10- d_2 -AA samples used 1/7 the values for the two β -protons at C10 and I=1.

Fig. 5.

HFEPR analysis of radicals in PGHS-1 reacted with AA and EtOOH. A) Solid line: field swept, echo-detected, 130 GHz EPR of PGHS-1 (80 μ M) premixed with 5 eq of AA and reacted anaerobically with 10 eq of EtOOH for 5 s at 24 $^{\circ}$ C before freeze-trapping. Dashed lines: summation of simulated spectra for the tyrosyl and pentadienyl radicals (9:1 ratio) shown in B and C. B: Simulated spectrum for a tyrosyl radical using the following parameters: $g_x=2.0070$, $g_y=2.0042$, and $g_z=2.0020$; full width half height Gaussian distribution of g about $g_x=0.003$. Principal hyperfine coupling values (in Gauss): two protons (9.5, 2.4, 6.2); two protons (1.0, 4.8, 0.8); one proton (6.7, isotropic); and one proton (7.8, isotropic). The Gaussian line-broadening function was set at 21 G full width half height. C) Simulated spectrum for a pentadienyl radical using parameters determined previously [24]: $g_x=2.0032$, $g_y=2.0032$, and $g_z=2.0020$. Principal hyperfine coupling values (in Gauss, all isotropic): two protons (10.4); two protons (3.3); two protons (16); one proton (11.5); one proton (9.8); and one proton (3.9). The Gaussian line-broadening function was set at 21 G full width half height. The radical concentration for spectrum C is 12% that of spectrum B.

Fig. 6. Cyclooxygenase active site structures for AA bound in a productive orientation to PGHS-1 (panel A; 1DIY [11]) and in reversed orientation to PGHS-2 (panel B; 1CVU [45]). Key residues are labeled and hydrogen bonds are indicated by dashed lines. The phenoxyl oxygen (orange) of Tyr385 (yellow) and the C13 (yellow) of AA (green) involved in H-transfer are highlighted to show the drastic distance change in the two panels.

Scheme 1.

Different AA-induced radical EPR signatures found in PGHS-1 and -2 during anaerobic single-turnover manual freeze EPR experiments.