Structural Characterization of Arachidonyl Radicals Formed by Aspirin-treated Prostaglandin H Synthase-2*

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Peroxide-generated tyrosyl radicals in both prostaglandin H synthase (PGHS) isozymes have been demonstrated to couple the peroxidase and cyclooxygenase activities by serving as the immediate oxidant for arachidonic acid (AA) in cyclooxygenase catalysis. Acetylation of Ser-530 of PGHS-1 by aspirin abolishes all oxygenase activity and transforms the peroxide-induced tyrosyl radical from a functional 33–35-gauss (G) wide doublet/wide singlet to a 26-G narrow singlet unable to oxidize AA. In contrast, aspirin-treated PGHS-2 (ASA-PGHS-2) no longer forms prostaglandins but retains oxygenase activity forming 11(\(^R\))- and 15(\(^R\))-hydroperoxyeicosatetraenoic acid and also retains the EPR line-shape of the native peroxide-induced 29–30-G wide singlet radical. To evaluate the functional role of the wide singlet radical in ASA-PGHS-2, we have examined the ability of this radical to oxidize AA in single-turnover EPR studies. Anaerobic addition of AA to ASA-PGHS-2 immediately after formation of the wide singlet radical generated either a 7-line EPR signal similar to the pentadienyl AA radical obtained in native PGHS-2 or a 26–28-G singlet radical. These EPR signals could be accounted for by a pentadienyl radical and a strained allyl radical, respectively. Experiments using 13(\(^d\))-AA, 13(\(^R\)\(^d\))-AA, 15d-\(^d\), 13,15d-\(^d\), and octadeterated AA (\(^d\)-AA) confirmed that the unpaired electron in the pentadienyl radical is delocalized over C11, C13, and C15. A 6-line EPR radical was observed when 16d-\(^d\)-AA was used, indicating only one strongly interacting C16 hydrogren. These results support a functional role for peroxide-generated tyrosyl radicals in lipoxygenase catalysis by ASA-PGHS-2 and also indicate that the AA radical in ASA-PGHS-2 is more constrained than the corresponding radical in native PGHS-2.

In recent years it has become evident that there are two distinct isoforms of prostaglandin H synthase (PGHS), the key enzyme in prostaglandin biosynthesis (1, 2). PGHS-1 is generally considered to play a housekeeping role, whereas PGHS-2 has been linked to various pathological processes (2, 3). Both PGHS isoforms have two catalytic activities; they are a cyclooxygenase activity that converts AA to PGG\(_2\) and a peroxidase activity that catalyzes the transformation of PGG\(_2\) to prostaglandin H\(_2\) (4). Aspirin irreversibly inhibits the cyclooxygenase activity by acetylation of a specific serine residue, Ser-530, in PGHS-1 and Ser-516 in PGHS-2 (5–8). Oxygenase activity is completely abolished in aspirin-treated PGHS-1 (ASA-PGHS-1), whereas aspirin-treated PGHS-2 (ASA-PGHS-2) still catalyzes formation of 15(\(^R\))- and 11(\(^R\))-HPETE (9–11) (Scheme 1). 15(\(^R\))-HPETE can be converted to epi-lipoxin, a potent anti-inflammatory mediator, via transcellular biosynthesis (12, 13). The mechanism of the lipoxygenase activity in ASA-PGHS-2 may, thus, provide insights into the pharmacology of the differential effect of aspirin on the two PGHS isoforms.

Substantial evidence supports the branched-chain radical mechanism for PGHS catalysis originally proposed by Ruf and co-workers (14, 15). In this mechanism, a specific protein-linked tyrosyl radical produced in the peroxidase cycle serves a catalytic role in cyclooxygenase activity. EPR observations in single turnover experiments provide convincing evidence that the peroxide-generated tyrosyl radicals in PGHS-1 and -2 can oxidize AA, the cyclooxygenase substrate, thus generating a pentadienyl AA radical characterized by a 7-line EPR spectrum (16–18). The tyrosyl radical mechanism explains the observed heme dependence of both enzyme activities and the requirement of the cyclooxygenase for a hydroperoxide activator (3, 19). The mechanism also is consistent with crystallographic data that show a tyrosine residue (Tyr-385 in PGHS-1 and Tyr-371 in PGHS-2) positioned between the heme and the AA binding site (20–23).

In PGHS-1, aspirin treatment causes a change of the peroxide-induced tyrosyl radical EPR spectrum from a 34–35-G doublet to a 25–26-G narrow singlet; this narrow singlet tyrosyl radical is unable to oxidize AA (16, 24). In contrast, the peroxide-induced tyrosyl radical in ASA-PGHS-2 retains the EPR characteristics of the peroxide-induced tyrosyl radical in native PGHS-2, a 29–30-G wide singlet (25). In the present study, we have examined whether the wide singlet tyrosyl radical of ASA-PGHS-2 is capable of AA oxidation to support the 11- and 15-lipoxygenase activity. The results indicate that the peroxide-induced tyrosyl radical in ASA-PGHS-2 does indeed react with AA to generate fatty acid radical intermediates. Interestingly, the AA radical species in ASA-PGHS-2 appears capable

EPR, electron paramagnetic resonance; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high performance liquid chromatography.

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§ The abbreviations used are: PGHS-1 and PGHS-2, prostaglandin H synthase isoform-1 and isoform-2; ASA-PGHS-2, PGHS-2 treated with aspirin; AA, arachidonic acid; ETOOH, ethyl hydroperoxide; PGG\(_2\), prostaglandin G\(_2\); d\(_{13}\)-AA, 5,6,8,9,11,12,14,15-octadeterated arachidionate; WS2, wide singlet radical found in peroxide-treated PGHS-2;
of adopting either a pentadienyl structure (as found with native PGHS-2) or a more constrained conformation (similar to that observed with native PGHS-1).

MATERIALS AND METHODS

AA was purchased from NuChek Preps, Inc., Elysian, MN. 5,6,8,9,11,12,14,15-Octadeuterated arachidonic acid (d8-AA) was obtained as a gift from Hoffmann-La Roche or purchased from Cayman Chemical Co. Mass analysis indicated that the d8-AA contained 24% heptadeuterated AA (16). d11-AAn, 13(R)-deuterated methyl ester (18, 52). Hemin and aspirin were obtained from Sigma. Aspirin was dissolved in ethanol immediately before use. Ethyl hydroperoxide (EtOOH) was a product of Polyscience Inc., Warrington, PA.

PGHS-1 was purified from sheep seminal vesicles (26), and human PGHS-2 was purified from recombinant material expressed in a baculovirus system (27). The holoenzymes were prepared by replenishing with heme; excess heme was removed by passage over DEAE-cellulose (28). Cyclooxygenase activity was determined by the rate of oxygen consumption (26). The batches of PGHS-1 used in this study had specific activities of 100–120 μmol of O2/min/mg; the PGHS-2 batches had specific activities of 20–42 μmol of O2/min/mg.

ASA-PGHS-2 was prepared as described previously (25). Briefly, the PGHS-2 holoenzyme was treated with aspirin, and the residual oxygenase activity was monitored periodically as the incubation proceeded at room temperature. When the oxygenase activity dropped to half the...
original value (reflecting the decreased oxygen consumption stoichiometry upon transition from cyclooxygenase to lipooxygenase catalysis), the reaction mixture was passed over a desalting column (10DG, Bio-Rad) to remove salicylate and unreacted aspirin. The use of a standardized oxygenase activity criterion for ending aspirin treatment was prompted by the observation of considerable variation in residual oxygenase activity after extended aspirin treatment among individual batches of ASA-PGHS-2. EPR spectra of the radical species obtained after the reaction of 11d-ASA, 13(R)d-ASA, and d₆-ASA fatty acid radical with oxygen and subsequent anaerobic addition of 1.5 eq of d₆-AA (Fig. 1, spectrum b and c). This radical displayed a 5-line EPR centered at g = 2.0027, with hyperfine splittings of 13.8−14.5 G. These EPR characteristics are very similar to those of the d₆-AA radical observed earlier in both PGHS-1 and -2 (16, 17). The samples trapped at 7 and 36 s after the addition of d₆-AA showed a similar radical species, with intensities of 0.2−0.3 spin/heme. Thawing and aerobic incubation of the samples with the d₆-AA-derived radical regenerated the WS2 tyrosyl radical (Fig. 1, spectra b’ and c’), consistent with formation of PGG₂ by reaction of the d₆-AA fatty acid radical with oxygen and subsequent reformation of the tyrosyl radical.

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Sequential Reaction of ASA-PGHS-2 with Hydroperoxide and Arachidonate—When ASA-PGHS-2 was reacted anaerobically with 5 eq of EtOOH, the EPR spectrum revealed a 29-G wide singlet (denoted WS2) (19) amounting to about 0.2 spin/heme (Fig. 1, spectrum a). A new radical was formed upon subsequent anaerobic addition of 1.5 eq of d₆-AA (Fig. 1, spectra b and c). This radical displayed a 5-line EPR centered at g = 2.0027, with hyperfine splittings of 13.8−14.5 G. These EPR characteristics are very similar to those of the d₆-AA radical observed earlier in both PGHS-1 and -2 (16, 17). The samples trapped at 7 and 36 s after the addition of d₆-AA showed a similar radical species, with intensities of 0.2−0.3 spin/heme. Thawing and aerobic incubation of the samples with the d₆-AA-derived radical regenerated the WS2 tyrosyl radical (Fig. 1, spectra b’ and c’), consistent with formation of PGG₂ by reaction of the d₆-AA fatty acid radical with oxygen and subsequent reformation of the tyrosyl radical.

The same procedure was used to trap AA-derived radicals during the reaction of 11d-AA, 15d-AA, 13(R)-d-AA, 13(R),15d₋₂-AA, and 16d₋₂-AA with the tyrosyl radical of ASA-PGHS-2. EPR spectra of the radical species obtained after anaerobic addition of each labeled AA to pre-formed tyrosyl radical are shown in Figs. 2 and 3. Substrate labeled with a single deuterium at positions 15,13(R) or 11 led to a sextet EPR signal (spectra B and C in Fig. 2 and spectrum B in Fig. 3, respectively), whereas the 13,15 double-labeled AA gave rise to a 5-line spectrum similar to that obtained for d₆-AA (compare spectra D and E in Fig. 2). The double-labeled 16d₋₂-AA yielded a 6-line radical EPR signal (Fig. 3, spectrum B), indicating that only one of the two β protons interacts strongly with the unpaired electron.

The results obtained using unlabeled AA were more complicated; representative data are shown in Fig. 4. The anaerobic addition of 1.5 eq of AA to ASA-PGHS-2 containing the WS2 tyrosyl radical (19) led to the replacement of the WS2 signal.

RESULTS

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corresponding proton coupling constant for each deuterium substitution. A coupling constant of 13 G was used for both C10
Dashed lines overlaid on the spectra represent computer simulations based on the parameters listed in Table I using 0.15 of the value of the corresponding proton coupling constant for each deuterium substitution. A coupling constant of 13 G was used for both C10 β-protons for simulation of the 16d2-AA radical. EPR conditions for the Bruker EMX spectrometer were: 2 G modulation amplitude; microwave frequency, 9.29 GHz; 1 mW power; time constant, 0.327 s; temperature, 110 K.

(Fig. 4, spectrum a) with a new signal very similar to that of WS2 (Fig. 4, spectrum b). This second radical EPR has an overall line width of 27 G centered at g = 2.0022 with similar total spin concentration to that of WS2. A sample freeze-trapped 24 s later exhibited a very similar EPR line shape but with substantially increased spin concentration (Fig. 4, spectrum c). These three samples subsequently were thawed and mixed with air; all three showed decreased radical intensity with little change in line-shape (Fig. 4, spectra a', b', c'). Spectra b and c in Fig. 4 are very different from the corresponding 7-line EPR spectrum obtained for control PGHS-2 sample (top spectrum in Fig. 6) (17). The lack of significant changes in the EPR spectrum after the addition of AA to ASA-PGHS-2 containing the tyrosyl radical (spectra b and c in Fig. 4) is unlikely to be due to oxygen contamination, because parallel experiments conducted under identical conditions using d8-AA clearly show the generation of an AA-derived radical (Fig. 1, spectra b and c). The experiment described in Fig. 4 was repeated several times with different batches of ASA-PGHS-2. A similar narrow 26–27-G EPR spectrum was obtained for samples trapped after AA addition in four of these replicate experiments. In one experiment, however, the narrow 27-G spectrum appeared initially after AA addition, with a 7-line EPR spectrum appearing subsequently (data not shown). In two other experiments, the addition of AA produced what seems to be a 7-line spectrum with a substantial amount of unconverted tyrosyl radical signal and perhaps some amount of the new narrow signal (spectrum A in Fig. 2). The variability evident in these results suggests that the radical generated from unlabelled AA in ASA-PGHS-2 can assume two different conformations, one producing a 27-G narrow singlet and the other producing a 7-line pentadienyl radical.

Power saturation studies were conducted for the 27-G singlet and both the 7- and 5-line EPR radical species. All three species relax more efficiently than isolated organic radicals, showing half-saturation power values around the milliwatt level at liquid nitrogen temperature (panel A in Fig. 5). The power dependence for the 7-line AA radical and 5-line d8-AA radical were determined for both the center and the immediately adjacent wing signals, with the wing signals showing a higher P1/2 value (panel B in Fig. 5). This is expected because the wing hyperfine lines have more contributions from nuclear spins to the relaxation (30). The saturation behavior of these three-carbon-centered radical species was very similar to that of the tyrosyl radical (panel A in Fig. 5).

Characterization of Carbon-centered Arachidonate Radicals and Analysis of EPR Spectra—The 7-line AA radical EPR spectra obtained with PGHS-2 or ASA-PGHS-2 are essentially identical and can be simulated using the parameter set published earlier (Table I) (17). Representative EPR data and the corresponding pentadienyl radical simulation are shown in Fig. 6 (top two traces). The parameter values producing optimal simulations are close to those of a prototypical pentadienyl radical (38) (fourth column, Table I). Our previous structural interpretation for this pentadienyl radical involves six distinct protons interacting strongly with the unpaired electron generated by initial hydrogen abstraction at the 13-pro-S position of AA (17). As illustrated at the top of Fig. 7, these six protons are located at C10, C11, C13, C15, and C16. We favored an assignment with two strongly interacting C10 β protons at dihedral angles of 32°/152° and one strongly interacting C16 β proton at a dihedral angle of 51°. This arrangement places C9 closer to C11 for endoperoxide formation and also brings C8 closer to C12 for subsequent ring closure. These assignments fix the conformation of the fatty acid skeleton from C9 to C17 of the carbon-centered substrate radical formed in the first step of the cyclooxygenase reaction with unlabelled AA in PGHS-2. When d8-AA was used, instead of obtaining a 7-line EPR, we observed
a 5-line EPR, characteristic of the pentadienyl d8-AA radical (Fig. 1, spectra b and c, and Fig. 2, spectrum E). This 5-line EPR is also optimally simulated using our previous parameters for the d8-AA radical intermediate found in native PGHS-2 (Table 1, third column) (17). The loss of one multiplicity in the spectra generated with the singly deuterated substrates are attributed to the loss of one hyperfine interaction with the protons at C11, C13(R), or C15. The same set of parameters used to fit the EPR data of unlabeled AA also reasonably simulated the EPR spectra obtained with the singly and doubly deuterated AAs when the value of the coupling constant for the deuterated position(s) was multiplied by 0.15 to account for the smaller nuclear gyromagnetic ratio of deuterium relative to hydrogen (Fig. 2, spectra B–E).

A pentadienyl radical is expected to have a large overall width (\(\approx 80\) G) and, thus, cannot adequately explain the narrow EPR spectrum of the unlabeled AA radical found with ASA-PGHS-2 (Fig. 4, spectra b and c). Attempts to deconvolute these narrow spectra as an arithmetic mixture of a pentadienyl radical and the WS2 tyrosyl radical were not successful due to the lack of wing features inherent in the EPR of the radical. The most likely alternative structures are conformations that limit electron delocalization and minimize the number of strongly interacting protons, thus decreasing the width of the signal. A good candidate is an allyl radical, with the unpaired electron delocalized over either C11-C13 or C13-C15. Such allyl radicals could arise if ASA-PGHS-2 bound the fatty acid in a conformation in which the C11-C12 and C14-C15 double bonds were not coplanar. With this model in mind and using the coupling constant values of typical allyl radical models as guidance (43), we performed computer simulations to obtain an optimal set of parameters to fit the data (Table I); the simulated allyl radical spectrum is shown in Fig. 6. Initial simulations using equal hyperfine interactions of the two endo protons (either the C11/C13 pair or the C13/C15 pair) and isotropic A tensor values failed to fit the data well. Changing from isotropic to anisotropic A tensors with a 3:1:2 ratio, as reported for other allyl radicals (31–33), led to significant improvement in the fit of the line shape but not the line width. Final adjustment to give unequal hyperfine interactions with the endo protons, with the proton at position 13 having a larger hyperfine value (\(A_{iso}/H = 15.3\) G) than the protons at position 11 or 15 (\(A_{iso}/H = 8.0\) G), led to the simulation shown in Fig. 6. Such an uneven spin-density distribution suggests a twisted allyl radical conformation. Interestingly, this same set of parameter values also closely simulates the narrow EPR spectrum found in PGHS-1 (Fig. 6, bottom spectrum), suggesting that this previously unexplained EPR signal (16) may also arise from a twisted allyl radical species.

The optimal values for the A tensors of the \(\beta\) protons derived from these simulations allowed calculation of the dihedral angle, \(\theta\), between the \(p_z\) orbital at C11 or C15 with the C10-H or C16-H bond, using the conventional McConnell relationship (33, 46),

\[ A_H = B_e \rho + B_1 \rho \cos^2 \theta \]  

(Eq. 1)

where \(A_H\) is the coupling constant due to hyperconjugation, \(\rho\) is the unpaired electron density at the terminal carbon of the pentadienyl radical, and \(B_e\) and \(B_1\) are constants (assigned values of 4 and 50 for a hydrocarbon free radical). The ratio of
A tensor value measured for the endo proton of the putative allyl radical (8.0 G) relative to that of a methyl proton in the ethyl radical (23 G) can be used to estimate a $^{1}H_{9252}/H_{9267}$ value of 0.35 (33). The dihedral angles associated with the strongly interacting $^{1}H_{9252}/H_{9267}$-protons at C10 or C16 can then be calculated to be 52° and 68°, respectively. The proposed conformations of the two possible allyl radicals are shown at the bottom of Fig. 7. Direct reaction of these intermediates with molecular oxygen would be expected to produce 11- or 15-HPETE.

**FIG. 5.** Power saturation behavior for the AA, $d_{8}$-AA, and tyrosyl radicals. Microwave power dependence of the 7-line AA radical generated by PGHS-2 (diamonds for center and triangles for the wing EPR features), the AA radical produced by ASA-PGHS-2 (solid squares), the $d_{8}$-AA radical generated by ASA-PGHS-2 (open squares for the center and open circles for the wing), and the tyrosyl radical (solid circles) at 95 K. Data were fitted by nonlinear regression (solid lines) as previously described (53) to the equation 

$$
\log(\frac{S}{P_{1/2}}) = -b/2 \log(P_{1/2} + P) + b/2 \log(P_{1/2}) + \log K,
$$

where $P_{1/2}$ is the power at half-saturation of the signal, and $b$ and $K$ are floating parameters. The optimal values obtained for $b$ are shown in the figure. The $P_{1/2}$ values obtained from nonlinear regression were 1.90 (center) and 3.54 (wing) mW for the PGHS-2 AA radical, 0.46 mW for the ASA-PGHS-2 AA radical, 1.24 (center) and 1.38 (wing) mW for the ASA-PGHS-2 $d_{8}$-AA radical, and 0.44 mW for the tyrosyl radical.

**TABLE I**

Parameters used to simulate the EPR spectra of AA radicals observed during reaction of PGHS tyrosyl radicals with unlabeled and deuterated AA

| Parameter | PGHS-2/ASA-PGHS-2 Model$^a$ | PGHS-1/ASA-PGHS-2 Model$^b$ |
|-----------|----------------------------|-----------------------------|
| $g$ factor | AA | $d_{8}$-AA | Pentadienyl | AA | $d_{8}$-AA | Allyl |
| Frequency, GHz | 9.236 | 9.238 | 9.2026 | 9.224 |
| Line width, GHz | 4 | 5 | 3.5 |
| A(H11/H15), G | 10.4 | 1.2 | 10.4 (H1/H5 exo) | 8.0 (12/4/8)$^c$ | 12.9 (endo) |
| A(H12/H14), G | 3.3 | 0.4 | 3.3 (H2/H4) | 3.0 |
| A(H13), G | 11.5 | 11.5 | 11.6 (H3) | 3.7 |
| A(H10 β1/β2), G | 16/16 | 16/16 | 9.6 (H1/H5 endo) | 15.3 (23/7.7/15.3) | 12.9 (endo) |
| A(H16 β1/β2), G | 9.5/3.9 | 9.5/3.9 | 8.0/4.5 |

$^a$ Data are from Davies et al. (38). The endo protons in the pentadienyl radical are not equivalent to the $\beta$ protons in the AA radical.

$^b$ Data are from Bascetta et al. (43). The endo protons in the AA allyl radical are either the H13/H15 or H11/H13 pairs. The exo protons are either the $\beta$ protons at C10 or C16.

$^c$ The A tensors of endo protons in the allyl radical are assumed to be anisotropic at a ratio of 3:1:2.

the $A$ tensor value measured for the endo proton of the putative allyl radical (8.0 G) relative to that of a methyl proton in the ethyl radical (23 G) can be used to estimate a $\rho$ value of 0.35 (33). The dihedral angles associated with the strongly interacting $\beta$-protons at C10 or C16 can then be calculated to be 52° and −68°, respectively. The proposed conformations of the two possible allyl radicals are shown at the bottom of Fig. 7. Direct reaction of these intermediates with molecular oxygen would be expected to produce 11- or 15-HPETE.

**Differences in the Chemical and Physical Properties of AA and $d_{8}$-AA**—The question arises why unlabeled AA and deuterated AA would give rise to different fatty acid radical signals. One possibility involves altered interactions between the fatty acids and the protein. The plausibility of such non-covalent isotope effects was evaluated by HPLC (Fig. 8). The retention time of $d_{8}$-AA was several minutes shorter than AA on a C$_{18}$ reversed-phase column, indicating that deuterated AA had weaker interactions with the hydrophobic column matrix.
The possibility of biochemical differences between AA and \(d_8\)-AA was further explored by measurement of their steady-state kinetic parameters for PGHS-1 cyclooxygenase activity. \(V_{\text{max}}\) and \(K_m\) values were determined by oxygen consumption assays using 20 nM PGHS-1 and either AA or \(d_8\)-AA as substrate at 30°C (Table II). Values of 0.54 ± 0.03 µM O_2/s and 4.6 ± 0.7 µM were obtained for \(V_{\text{max}}\) and \(K_m\) when AA was the substrate, whereas 0.50 ± 0.03 µM O_2/s and 5.0 ± 0.7 µM were obtained as \(d_8\)-AA was the substrate. Similar parameter values were also obtained for \(13^{(R)}d\)-AA and \(15d\)-AA (Table II). Isotope replacement, thus, had little effect on the steady-state cyclooxygenase kinetics.

**DISCUSSION**

Oxidation of AA by Peroxide-induced ASA-PGHS-2 Radical(s)—Oxidation of AA to a fatty acid radical is a key step in the proposed branched-chain mechanism for PGHS cyclooxygenase catalysis (14, 15). It has been previously demonstrated that the peroxide-induced tyrosyl radicals characterized by a wide doublet (33–35 G) or wide singlet (33–35 G) in PGHS-1 and a wide singlet (29–30 G) in PGHS-2 can oxidize AA to produce a carbon-centered AA radical (16–18). The alternative tyrosyl radical characterized by a narrow singlet (25–26 G) formed in PGHS-1 treated with cyclooxygenase inhibitors or in PGHS-1 with the Y385F mutation was not able to oxidize AA to form the AA derived radical (16, 17, 34).

The action of aspirin is very different in PGHS-2 from that in PGHS-1. ASA-PGHS-2 retains oxygenase activity although the products are 11- and 15-HPETE instead of PGG_2 (i.e. lipoxygenase-like products), and ASA-PGHS-2 treated with peroxide forms the same wide singlet (29–30 G) tyrosyl radical seen with in PGHS-2 itself (17, 25). When PGHS-2 oxygenase activity is completely blocked by complexation with nimesulide or by introduction of the Y371F mutation an alternative narrow singlet tyrosyl radical (21–23 G) is formed (24, 25).

With a link between the wide singlet tyrosyl radical and oxygenase capacity established in PGHS-2 and ASA-PGHS-2, it was important to test the ability of the wide singlet radical in ASA-PGHS-2 to actually carry out the oxidation of AA required in lipoxygenase catalysis. The present results show that the 29–30 G wide singlet in ASA-PGHS-2 is indeed able to oxidize AA to generate an AA-derived radical (Figs. 1–3). The EPR line-shape changes observed with ASA-PGHS-2 in the AA radical upon deuterium substitution are almost identical to those found for the same substrates with native PGHS-2 (Fig. 1 in Peng et al. (18)). A smaller coupling constant for the \(^{1}H\)-protons at C10 was needed to achieve adequate simulation of the signal produced by \(16d_2\)-AA, indicating that the spin-density at C11 and C15 may not be equal. The 6-line signal observed upon incubation of ASA-PGHS-2 with \(16d_2\)-AA indicates that there is only one strongly interacting \(^{1}H\)-proton at C16, similar to that observed with the AA radical in PGHS-2 (52).

The oxygenase activities in PGHS-2 and ASA-PGHS-2 give rise to very different products. Given the similarity in the EPR signals of the radicals produced with deuterated AA substrates, the mechanistic divergence in the two enzymes must occur at some point after AA radical formation. This is consistent with analyses of the stereochemistry of hydrogen atom abstraction at C13 and oxygen addition at C15 and with the \(K_m\), AA values for ASA-PGHS-2 and PGHS-2, all of which indicate that the methyl terminus of AA adopts an altered conformation when Ser-516 is acetylated (25, 35, 36).

**Structure of the Arachidonic Acid Carbon-centered Radical in ASA-PGHS-2**—The finding of two distinct types of EPR signal for the carbon-centered radical in ASA-PGHS-2 with
unlabeled AA but only one signal in experiments with labeled AAs complicates data interpretation. With d8-AA as substrate the AA radical has the same pentadienyl conformation in both PGHS-2 and ASA-PGHS-2. However, with unlabeled AA the pentadienyl radical was observed only in some of the samples, the conversion from tyrosyl radical to AA radical was often incomplete (Fig. 2, spectrum A), and a signal that can be simulated as a twisted allyl radical was frequently observed (Fig. 4 and Table I).

Allyl radicals containing various electron-withdrawing groups at the exo position show minimal perturbation of the electron density distribution (37). The isotropic hyperfine value for the two endo-protons at C11/C15 in the putative ASA-PGHS-2 AA allyl radical is much smaller than expected from allyl radical models (Table I) (37, 38). Nelson et al. (33) did observe an allyl radical intermediate in purple lipoxygenase with hyperfine coupling constants of the endo protons 2–3G smaller than a typical allyl radical model. The structural implication of that observation was not entirely clear, although interaction of an adjacent double bond with the ferrous center or addition of oxygen to this double bond were suggested as possible explanations. Such mechanisms do not apply in our case because the heme center is distant from the site of AA radical formation, and the present experiments were conducted under anaerobic conditions.

In view of the unexpected adjustments of hyperfine coupling constants that were required to simulate the observed EPR data, we have examined the consequences of structural distortions on the spin density distribution in the related 1,3-dimethyl allyl radical using density functional theory calculations. As expected, when the allyl radical is planar, the spin densities on carbons 2 and 4 are identical (Fig. 9, dihedral angle of 0°); this planar conformation is the structure obtained after geometry optimization. When the radical is distorted away from planarity by rotation about the C3-C4 bond, the spin densities vary systematically as the dihedral angle is increased to 90°, with the density on C2 decreasing and that on C4 increasing (Fig. 9). At 90°, there is almost no unpaired spin on C2 and a large, positive spin density on C4. A
similar result was obtained in a calculation using a C11-C13 arachidonic acid allyl radical. In this case, it is the spin density on C11 and C13 that is sensitive to the C11–12–13–14 dihedral angle. The density at C11 (C13) is almost at a maximum (minimum) in the conformation found in the crystallographic structure of the PGHS-1-AA complex (23) and essentially zero when the dihedral angle is rotated 90° away from this conformation. The spin densities on the other carbon atoms change only slightly over this range of rotation. A dihedral angle near 45° matches the spin density distribution needed to account for the putative allyl radical obtained with unlabeled AA (Table I and Fig. 7). The ratio of the spin-density at C2 and C4 computed for a 45° dihedral (0.53, Fig. 9) is almost identical to the ratio of the proton coupling constants between C11 and C13 (or C15 and C13) (0.52, Table I). This result provides theoretical support for the hypothesis of a strained allyl radical generated in ASA-PGHS-2 using unlabeled substrate.

Crystallographic data indicate that the volume of the cyclooxygenase substrate channel is substantially smaller in PGHS-1 than in PGHS-2 (4, 20–23). Mutational analysis indicates this additional constriction in the PGHS-1 channel is the result of amino acid differences at positions 434, 513, and 523 (41). This difference in the size of the active site can account for the effects of acetylation of Ser-530 on oxygenase activity; activity is abolished in PGHS-1 but retained in PGHS-2. We propose that the observation of a putative allyl radical in native

![Fig. 8. Interactions of AA and d8-AA with chromatographic support. The fatty acids were dissolved in methanol and injected individually (panels A and B) or as a 1:1 mixture (panel C) onto a reversed-phase HPLC column (Varian Microsorb MV C18, 4.6 × 250 mm) and eluted with acetonitrile:H2O:acetic acid (58:42:0.2 v/v/v) at 2 ml/min. The absorbance at 220 nm was monitored with an HP1100 diode array detector.](image)

**TABLE II**

| Parameter* | AA | 13(R)d-AA | 15d AA | d8-AA |
|------------|----|-----------|--------|-------|
| \(K_m\), \(\mu M\) | 4.6 ± 0.7 | 5.0 ± 0.8 | 3.8 ± 0.6 | 5.0 ± 0.7 |
| \(V_{max}\), \(\mu M O_2/s\) | 0.54 ± 0.03 | 0.50 ± 0.02 | 0.51 ± 0.02 | 0.50 ± 0.03 |

* Oxygen consumption was determined at 30 °C (26) using 20 nM PGHS-1 subunit and substrate levels varying from 1 to 40 \(\mu M\). The parameter values (± S.D.) were obtained from nonlinear regression fitting to the Michaelis-Menten equation.
Arachidonyl Radicals Formed by Aspirin-treated PGHS-2

PGHS-1 but not native PGHS-2 is another indication of the greater steric restrictions in the PGHS-1 cyclooxygenase site. A narrow EPR spectrum derived from an AA radical has been typically observed with PGHS-1 (Ref. 16 and the bottom spectrum in Fig. 6), and this narrow EPR signal can be simulated well using the same parameter set used to simulate the putative allyl AA radical EPR in ASA-PGHS-2 (Table I). In the latter case, it would be the presence of an acetyl group on Ser-516 that restricts the conformational space accessible to the radical intermediate, resulting in an allyl radical rather than the pentadienyl radical observed with unmodified PGHS-2. This perturbation of the conformation of bound AA in ASA-PGHS-2 is presumably also the reason for the generation of lipoxygenase products rather than prostanoids. Our EPR data suggest that three possible carbon-centered radical intermediates may be formed, as illustrated in Fig. 7; they are a pentadienyl radical and two twisted allyl radicals with electron delocalization occurring over either C11–C13 or C13–C15, leading to either 11-HPETE or 15-HPETE, respectively, in the presence of oxygen. Both products are in fact formed by ASA-PGHS-2, with 15-[(R)]-HPETE as the predominant product (25).

Formation of 15-[(R)]-HPETE by ASA-PGHS-2 is initiated by abstraction of the 13-pro S hydrogen atom, identical to the first step of the cyclooxygenase reaction in PGHS-1 and PGHS-2 (25, 35). Rotation of the ω side arm of AA around the C13–C14 bond in response to acetylation of Ser-516 has been proposed to account for the reversed stereochemistry of the oxygenation at C15 (35). If ω side arm rotation occurs, then the pentadienyl radical we observe in ASA-PGHS-2 would not have two exo protons at C11 and C15 as in PGHS-2 and PGHS-1. Instead it would have one exo (C11) and one endo proton (C15), and the hydrogen coupling constants for the entire conjugated system would be somewhat different. However, these changes are minor in the reported spectra of pentadienyl radicals in solution with the coupling constants of all protons in both isomers within one gauss (44). Such changes will not be easily distinguished from the pentadienyl radical EPR observed for untreated PGHS-2 due to the large line width in powder spectra such as those reported here. The changes in product profile seen with the PGHS-1 V439L mutant and other cyclooxygenase site mutants have also been attributed to perturbations of the conformation of bound AA (4, 39). It is worth noting that most of these mutants yield products that lack the endoperoxide and the cyclopentane structural elements, suggesting that the AA conformation leading to PGG2 formation is easily disrupted by active site modifications. More recent examination of the Ser-530 and Val-349 mutants in both COX-1 and COX-2 further substantiates the critical role of these residues in conferring the stereoselectivity of the oxygenation at C15 (40).

Isotope Effects on AA Radical Conformation—In both the current study (Fig. 1) and our previous single-turnover EPR studies (16–18), a 5-line pentadienyl radical EPR was always obtained when d8-AA was reacted with PGHS-1, PGHS-2, or ASA-PGHS-2 under the conditions used in the present study. In contrast, unlabeled AA leads to a putative distorted allyl AA radical in PGHS-1 and ASA-PGHS-2 and a pentadienyl radical in unmodified PGHS-2 (Fig. 6). If the hypothesis of an allyl radical is correct, then the greater tendency to form a constrained substrate radical with AA than with d8-AA suggests that the proteins have a different interaction with unlabeled substrate, stabilizing the thermodynamically disfavored twisted allyl radical. To place the required stabilization in a more quantitative context, the barrier of rotation to take one of the π-systems out of conjugation has been reported to be 11.7 kcal/mol for the parent pentadienyl radical in solution (48). The initial penalty for rotation of a 1,5-disubstituted radical with both substituents in the endo position may be somewhat lower than for the parent pentadienyl radical because of relief of unfavorable steric interactions (1,3-strain), but it is likely to still be considerable. In addition, the twisting of the allyl radical will require an energetic penalty. In our theoretical 1,3-dimethyl allyl radical model the energy of the conformer with a dihedral angle of 45° lies 11 kcal/mol above the planar conformation and at a 90° angle; the calculated energy was 22 kcal/mol above the planar radical. This latter value is much larger than that reported for the rotational barrier in the parent allyl radical we observe in ASA-PGHS would not have two exo protons at C11 and C15 as in PGHS-2 and PGHS-1. Instead it would have one exo (C11) and one endo proton (C15), and the hydrogen coupling constants for the entire conjugated system would be somewhat different. However, these changes are minor in the reported spectra of pentadienyl radicals in solution with the coupling constants of all protons in both isomers within one gauss (44). Such changes will not be easily distinguished from the pentadienyl radical EPR observed for untreated PGHS-2 due to the large line width in powder spectra such as those reported here. The changes in product profile seen with the PGHS-1 V439L mutant and other cyclooxygenase site mutants have also been attributed to perturbations of the conformation of bound AA (4, 39). It is worth noting that most of these mutants yield products that lack the endoperoxide and the cyclopentane structural elements, suggesting that the AA conformation leading to PGG2 formation is easily disrupted by active site modifications. More recent examination of the Ser-530 and Val-349 mutants in both COX-1 and COX-2 further substantiates the critical role of these residues in conferring the stereoselectivity of the oxygenation at C15 (40).

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radical (17.4 kcal/mol), obtained using B3LYP density functional theory calculations (49). The larger energy barrier may be a consequence of two methyl substituents on carbons 2 and 4 in the 1,3-dimethylallyl radical model. An increase in the stabilization energy of the allyl radical by methyl substitution has indeed been reported (50). Unfortunately, more accurate experimental measurements of stabilization energy developed since this report (51) have not been applied to allyl radicals substituted with methyl groups to verify this conclusion.

Deuterium isotope effects in the absence of covalent bond breaking and formation have been extensively documented (42). Noncovalent deuterium isotope effects are primarily attributed to the shorter bond length of C-D versus C-H as well as to the changes in molecular volume and polarity or polarizability. Increasing deuterium substitution in hydrocarbons usually increases their polarity and, thus, hydrophilicity, and our HPLC results support such an effect in AA (Fig. 8). The difference in affinity for the C18 stationary phase between AA and d8-AA may be relevant considering the hydrophobic nature of the cyclooxygenase substrate binding site, which mainly consists of nonpolar amino acid residues (20–23). One interpretation is that the lower hydrophobicity of d8-AA compared with AA provides decreased interaction with the protein and, thus, increased flexibility once the fatty acid is bound. This decreased constraint favors formation of a pentadienyl radical, whereas stronger binding interactions and higher constraint with unlabeled AA favor an allyl radical, especially when the binding site becomes more restricted, as in PGHS-1 or ASA-PGHS-2 (20–23, 41). However, this interpretation finds little support in the steady-state kinetic data, where single or multiple deuteration showed little effect on the arachidonate K_m values or the V_max of the cyclooxygenase activity. Clearly, the lack of change of K_m does not rule out binding affinity changes because the cyclooxygenase K_m value may be dominated by factors other than binding; direct binding measurements are required to resolve this issue.

In summary, the present results show that the peroxide-induced tyrosyl radical in ASA-PGHS-2 is capable of oxidizing arachidonate to a carbon-centered fatty acid radical. This provides the first direct support for a tyrosyl radical mechanism of lipoxigenase catalysis in ASA-PGHS-2. Computer simulations of the EPR spectra suggest plausible structures for the arachidonyl radicals formed in ASA-PGHS-2 and in PGHS-1; these include a distorted allyl radical and a pentadienyl radical.

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