Analysis of Hydroperoxide-Induced Tyrosyl Radicals and Lipoxygenase Activity in Aspirin-Treated Human Prostaglandin H Synthase-2†

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ABSTRACT: A hydroperoxide-induced tyrosyl radical has been proposed as a key cyclooxygenase intermediate for the "basal" isoform of prostaglandin H synthase (PGHS-1). In the present study with the "inducible" isoform (PGHS-2), hydroperoxide was also found to generate a radical in high yield, a wide singlet at $g = 2.0058$ (29 G peak to trough). Reaction of PGHS-2 with a tyrosine-modifying reagent, tetranitromethane (TNM), resulted in cyclooxygenase inactivation and a much narrower radical EPR signal (22 G peak to trough). Addition of a cyclooxygenase inhibitor, nimesulide, similarly resulted in a narrow PGHS-2 radical. In PGHS-1, cyclooxygenase inhibition by tyrosine nitration with TNM or by active site ligands leads to generation of a narrow EPR instead of a wide EPR, with both signals originating from authentic tyrosyl radicals, indicating that the hydroperoxide-induced radicals in PGHS-2 are also tyrosyl radicals. Treatment of PGHS-2 with aspirin (acetyl salicylic acid, ASA) was previously shown to result in acetylation of a specific serine residue, cyclooxygenase inhibition, and increased lipoxygenase activity. Acetylation of PGHS-1 by ASA, in contrast, inhibited both lipoxygenase and cyclooxygenase activity. We now have found the ASA-treated PGHS-2 radical to be indistinguishable from that in control PGHS-2. Addition of nimesulide to ASA-treated PGHS-2 inhibited the lipoxygenase and resulted in a narrow radical EPR like that seen in PGHS-2 treated with TNM or nimesulide alone. Retention of PGHS-2 oxygenase activity was thus associated with retention of the native radical, and loss of activity was associated with alteration of the radical. Both native and ASA-treated PGHS-2 produced only the *R* stereoisomer of 11- and 15-HETE, demonstrating that the lipoxygenase stereochemistry was not changed by ASA. Native and ASA-treated PGHS-2 had lipoxygenase K_m values considerably higher than that of the control PGHS-2 cyclooxygenase. Taken together, these results suggest that the same PGHS-2 tyrosyl radical serves as the oxidant for both cyclooxygenase and lipoxygenase catalysis and that acetylation of PGHS-2 by ASA favors arachidonate binding in an altered conformation which results in abstraction of the *pro-R* hydrogen from C13 and formation of 11(*R*)- and 15(*R*)-HETE.

Prostaglandin H synthase (PGHS[1]) catalyzes a key step in prostanoid biosynthesis (Samuelsson et al., 1978). Two isoforms of PGHS have been described. PGHS-1 is believed to be a housekeeping enzyme, and PGHS-2 is believed to play important roles in cell proliferation and inflammation (Herschman, 1996). Human PGHS-1 and PGHS-2 have about 60% identity at the amino acid level (Hla & Neilson, 1992).

Both isoforms of PGHS have cyclooxygenase and peroxidase activity (Fletcher et al., 1992). Reaction of PGHS-1 with hydroperoxide initially produces a tyrosyl radical with a wide doublet EPR signal (Kulmacz et al., 1987; Karthein et al., 1988; DeGray et al., 1992). This radical is present during cyclooxygenase catalysis (Tsai et al., 1992) and is capable of oxidizing arachidonic acid to an arachidonyl radical (Tsai et al., 1995). Mutagenesis studies indicate that the wide doublet tyrosyl radical in PGHS-1 is likely to reside on Tyr385 (Shimokawa et al., 1990; Tsai et al., 1994). Tyr385 is located a short distance from the heme in PGHS-1, positioned between the heme and the putative fatty acid binding site (Picot et al., 1994). This evidence supports the proposal by Ruf and colleagues (Karthein et al., 1988) that a tyrosyl radical generated by initial reaction with hydroperoxide acts as the key oxidant in cyclooxygenase catalysis. PGHS-2 has a tyrosine (Tyr371) in the position corresponding to Tyr385 in PGHS-1 (Hla & Neilson, 1992). Reaction of PGHS-2 with hydroperoxide gives rise to a free radical (Hsi et al., 1994), but there is little published evidence that the PGHS-2 radical is also a tyrosyl radical.

Treatment of PGHS-1 with ASA results in the acetylation of Ser530, loss of cyclooxygenase activity (van der Ouderaa

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[1] Abbreviations: PGHS-1 and PGHS-2, prostaglandin H synthase isoforms 1 and 2; ASA, acetyl salicylic acid (aspirin); 11- and 15-HETE, 11- and 15-hydroxyeicosatetraenoate; 11- and 15-HPETE, 11- and 15-hydroperoxyeicosatetraenoate; PGG_2 and PGH_2, prostaglandins G_2 and H_2; TNM, tetranitromethane; DNBPG, (*R*)-(−)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine.

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et al., 1980; Roth et al., 1983), and formation of a narrow singlet tyrosyl radical which is incapable of oxidizing arachidonate (Kulmacz et al., 1991; Tsai et al., 1995). ASA has been found to acetylate the corresponding residue in PGHS-2, Ser516 (Lecomte et al., 1994; Wennogle et al., 1995). In contrast with PGHS-1, where acetylation destroys all oxygenase activity, the loss of cyclooxygenase activity in ASA-treated PGHS-2 is accompanied by an increase in lipoxygenase activity (Holtzman et al., 1992; Meade et al., 1993; Lecomte et al., 1994; O'Neill et al., 1994). The 15-(*R*)-HPETE produced by ASA-treated PGHS-2 has a configuration at C15 opposite of that in PGG_2 (Holtzman et al., 1992; Lecomte et al., 1994; O'Neill et al., 1994), prompting a proposal that acetylation of Ser516 alters the stereoselectivity of oxygen addition (Lecomte et al., 1994).

The retention of oxygenase activity in ASA-treated PGHS-2 provides an important test of the proposed role of hydroperoxide-induced radical as the catalytic oxidant. We have examined the effect of ASA treatment on the EPR spectrum of the radical in human PGHS-2. The results indicate that ASA-treated PGHS-2 has the same tyrosyl radical available to serve as the oxidant in 15-lipoxygenase catalysis as that available for cyclooxygenase catalysis in native PGHS-2. In addition, stereochemical analyses of 11-HETE and 15-HETE show that acetylation of PGHS-2 does not change the stereochemistry of oxygen addition in lipoxygenase catalysis. This has led to our proposal that arachidonate can be bound in two distinct conformations in the cyclooxygenase active site, one leading to formation of PGG_2 and the other to formation of HPETEs.

MATERIALS AND METHODS

Materials. Glycerol, guaiacol, hydrogen peroxide, hemin chloride, D-tryptophan, TNM, and ASA were from Sigma Chemical Co. (St. Louis, MO). PD-10 desalting columns were from Supelco (Bellefonte, PA). Ethyl hydroperoxide was from Polysciences (Warrington, PA). Nimesulide, 11-(*S*)-HETE, 11(*RS*)-HETE, 15(*S*)-HETE, and 15(*RS*)-HETE were obtained from Cayman Chemical Co. (Ann Arbor, MI). Anhydrous pyridine and 1-naphthoyl chloride were from Aldrich (Milwaukee, WI). Arachidonic acid was obtained from NuChek Preps (Elysian, MN) and treated with sodium borohydride to decompose hydroperoxides (Kulmacz & Lands, 1987).

PGHS-2 Expression and Purification. Recombinant human PGHS-2 was expressed in cultured insect cells and purified to homogeneity as the apoprotein essentially as described previously (Kulmacz & Wang, 1995; Wennogle et al., 1995). Holoenzyme was reconstituted by addition of heme [1.4 mol/(mol of subunit)], treated with DEAE-cellulose to remove excess heme, and subjected to gel filtration chromatography on a PD-10 column to exchange the buffer (Kulmacz et al., 1987). Ovine PGHS-1 was purified to homogeneity from sheep seminal vesicles (Kulmacz & Lands, 1987).

Protein concentrations were assayed as described by Peterson (1983). Heme content was determined fluorimetrically (Morrison, 1965; Sassa, 1976) using purified ovine PGHS-1 as the standard. Oxygenase activity was measured from the kinetics of oxygen uptake determined with an oxygen electrode (Kulmacz & Lands, 1987) at 30 °C. Oxygenase activity thus includes both cyclooxygenase (2 mol of oxygen consumed per mole of arachidonate) and lipoxygenase (1 mol of oxygen consumed per mole of arachidonate) activities. The arachidonate oxygenation activity contributed by cyclooxygenase catalysis can be estimated by subtracting the lipoxygenase activity from the oxygenase activity (both in terms of oxygen consumed per minute per milligram of protein) and dividing the result by 2. The standard oxygenase reaction mixture contained 0.1 M potassium phosphate (pH 7.2), 5 mM D-tryptophan, 0.1 mM arachidonate, and 1 μM heme. One unit of oxygenase produces a peak velocity of 1 nmol of oxygen/min. Lipoxygenase activity was assayed spectrophotometrically at 23 °C using the absorbance increase at 235 nm during the first few seconds of the reaction and an extinction coefficient of 23.3 mM^{-1} cm^{-1} (Graff et al., 1990). Decomposition of newly formed prostaglandin endoperoxides to 12-hydroxyheptatrienoic acid should be minimal over such short reaction times in the absence of glutathione (Capdevila et al., 1995), and so the initial absorbance changes reflect primarily lipoxygenase product formation. The standard lipoxygenase reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 1 mM D-tryptophan, and 60 μM arachidonate at 23 °C. Peroxidase activity was measured spectrophotometrically at 436 nm (Ohki et al., 1979) in a reaction mixture containing 0.1 M Tris-HCl (pH 8.0), 0.5 mM guaiacol, 0.4 mM hydrogen peroxide, and 1 μM heme. One unit of peroxidase produces a velocity of 1 nmol of hydrogen peroxide/min.

ASA-Treated PGHS-2. To prepare stable samples of ASA-treated PGHS-2, the holoenzyme (14 μM subunit) and ASA (0.8 mM) were reacted at room temperature in 0.1 M potassium phosphate (pH 7.2) containing 0.1% Tween-20. The final ethanol level was 2%. Aliquots were withdrawn periodically for enzyme assay as described above or for reisolation of the synthase by gel filtration on a PD-10 column at 4 °C in the same buffer. Samples were concentrated by ultrafiltration on a YM30 membrane (Amicon) before addition of glycerol to 20%. Oxygenase and lipoxygenase K_m values for native and ASA-treated PGHS-2 were determined at 25 °C in buffer containing 0.1 M Tris (pH 8.0), 5 mM D-tryptophan, 0.1% Tween-20, and 0.4−80 μM arachidonate. Reactions were started by addition of enzyme and monitored by either oxygen consumption (oxygenase) or absorbance changes at 235 nm (lipoxygenase) as described above. Data were fitted to the conventional Michaelis−Menten equation using a nonlinear least-squares routine to estimate the values of K_m and V_{max}.

EPR Spectroscopy. EPR spectra were recorded with a Varian E-6 spectrometer equipped with transfer lines for liquid helium or liquid nitrogen cooling as described previously (Kulmacz et al., 1987). Enzyme samples were diluted in 0.1 M potassium phosphate (pH 7.2) containing 0.1% Tween-20 or 0.05% octyl glucoside and 20−25% glycerol. For reaction with EtOOH, the sample was chilled in a bath at 0 °C before addition of 2−6 μL of an aqueous solution of the hydroperoxide with a microsyringe and immediate mixing with a nichrome wire loop. The reaction was stopped by freezing the sample in a dry ice/acetone bath, and the samples were stored in liquid nitrogen until EPR analysis. For extended reaction, individual samples were thawed and incubated at 0 °C for the desired time before freeze-quenching and EPR analysis. The radical intensities, obtained by double integration and comparison to a copper

Table 1: Hyperfine Coupling Constants Used for Computer Simulation of the PGHS-2 Radical EPR Signal

carbon atom	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)
β1	14	14	14
β2	3	3	3
C3	9.3	2.5	6.9
C5	9.3	2.5	6.9
C2	6	5	4
C6	6	5	4

sulfate standard (Aasa & Vanngard, 1975), are given in units of spins per heme.

Computer simulation of the PGHS-2 radical with a modified POWFUN program (Hoganson & Babcock, 1992; Tsai et al., 1995) used a Gaussian line shape with a 3 G line width, $g_x = 2.0068$, $g_y = 2.004$, $g_z = 2.0023$, and a frequency of 9.307 GHz. The hyperfine coupling constants employed (Table 1) were empirically adjusted from values determined from ENDOR spectra of the PGHS-1 wide doublet tyrosyl radical (W. Shi, C. W. Hoganson, G. T. Babcock, G. Palmer, R. J. Kulmacz, and A.-L. Tsai, unpublished results). The major adjustment was decreasing the value of the coupling constant for the β1 proton from 21 to 14 G; the values for the protons at ring positions 3 and 5 were not changed from those in the PGHS-1 wide doublet. Basing the parameter set on observed characteristics of the PGHS-1 tyrosyl radical was expected to be more relevant to simulation of the PGHS-2 radical than using parameter sets of radicals formed from free tyrosine in crystals or in solution.

HETE Stereochemistry. Analysis of the stereochemistry of 11- and 15-HETE produced by PGHS-2 and ASA-treated PGHS-2 utilized chiral phase HPLC of naphthoyl derivatives of the methyl esters of the HETEs (Hawkins et al., 1988). PGHS-2 holoenzyme (5 μM subunit) was preincubated with or without 0.8 mM ASA in 1.6 mL of 0.1 M potassium phosphate (pH 7.2) and 5 mM tryptophan for 20 min at room temperature. Arachidonate (100 μM) was added and the reaction allowed to proceed for 3 min. The lipid products were extracted with 2 volumes of ice-cold diethyl ether/methanol/1 M citric acid and dried over anhydrous sodium sulfate. To isolate 11- and 15-HETE, the extract was separated on a C18 reversed phase column with acetonitrile/water/glacial acetic acid (100:100:0.05) as the solvent; eluting compounds were monitored by absorbance at 235 nm and the HETE peaks identified by coinjection with standards. The two HETE fractions were subsequently handled separately.

After addition of ricinoleic acid as carrier, the HETE fractions were lyophilized and converted to the methyl esters by reaction with diazomethane. The methyl esters were purified by HPLC on a C18 reversed phase column with methanol/water (9:1) as the solvent, monitored by absorbance at 235 nm. Ricinoleic acid carrier was added to each purified HETE methyl ester solution, the solvent evaporated, and the residue dissolved in 30 μL of pyridine and 3 μL of 1-naphthoyl chloride. After reaction for 30 min at room temperature, the solvent was evaporated and the residue extracted with hexane. The hexane-soluble products were dried with a stream of nitrogen and dissolved in methanol/water (9:1), and the naphthoyl derivatives of the HETE methyl esters were purified by reversed phase HPLC as described above for the methyl esters themselves. Identification of 11- and 15-HETE was confirmed by their comigration with authentic standards at every stage of the derivatization process. The naphthoylated methyl esters were finally analyzed by HPLC on a covalent DNBPG chiral phase column (Bakerbond RP-7113; 4.6 × 250 mm) eluted at 1 mL/min with hexane/2-propanol (100:0.15), with detection at 235 nm. The order of elution of the *R* and *S* isomers was confirmed by coinjection of the naphthoylated derivative of standard 11(*S*)-HETE or 15(*S*)-HETE with the corresponding racemic mixture.

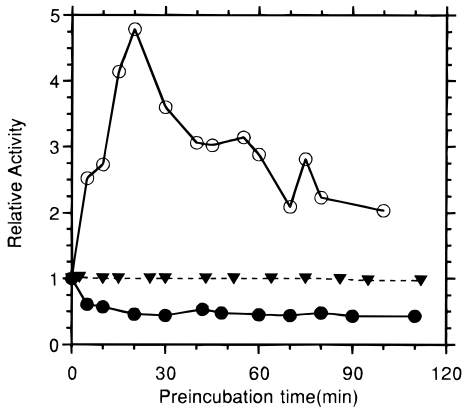


FIGURE 1: Kinetics of changes in hPGHS-2 oxygenase and lipoxygenase activities during preincubation with ASA at room temperature. ASA was added in 0.8 mM increments to PGHS-2 holoenzyme (14 μM subunit) at 0, 46, and 73 min. Aliquots were removed for assay of oxygenase (filled circles) and lipoxygenase (open circles) activity as described in Materials and Methods. Oxygenase activities from a control incubation without ASA are represented by filled triangles. Activities were normalized to the initial control values, which were as follows: oxygenase, 18.1 μmol of O_2 min^{-1} (mg of protein)$^{-1}$; and lipoxygenase, 0.47 μmol of O_2 min^{-1} (mg of protein)$^{-1}$.

RESULTS

Kinetics of Cyclooxygenase and Lipoxygenase Activity Changes during Incubation of PGHS-2 with ASA. Preincubation of PGHS-2 with ASA resulted in a time-dependent loss of about half of the oxygenase activity, as measured with the oxygen electrode (Figure 1), as expected from a previous report (Lecomte et al., 1994). More than 95% of the oxygenase activity remained even after 110 min of incubation in the absence of ASA (Figure 1), demonstrating the stability of the enzyme itself. The lipoxygenase activity increased steadily over the first 20 min of reaction with ASA, reaching about 5 times the initial rate (Figure 1). When calculated on the basis of arachidonate consumption, the lipoxygenase activity made up 5% of the oxygenase (i.e., cyclooxygenase plus lipoxygenase) activity initially and 42% of the oxygenase activity after 20 min of ASA treatment. Preincubation with ASA for longer than 20 min led to a decrease in the lipoxygenase activity, to a level about twice the initial value (Figure 1). PGHS-2 peroxidase activity was unaffected by incubation with ASA (data not shown), indicating that the changes in oxygenase and lipoxygenase activities were due to actions at the cyclooxygenase active site, and not at the peroxidase active site.

Changes in lipoxygenase activity during preincubation of PGHS-2 with ASA were also monitored by chromatographic analysis of the capacity for 11- and 15-HETE formation (Figure 2). ASA treatment for 10 or 20 min produced coordinate increases in subsequent synthesis of the two HETEs from arachidonate; longer ASA treatment led to a

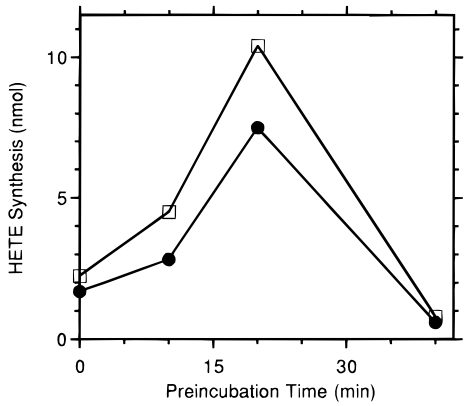


FIGURE 2: Effects of ASA preincubation on production of 11-HETE and 15-HETE by PGHS-2. PGHS-2 holoenzyme (5 μM subunit) was preincubated with 0.8 mM ASA in 0.1 M potassium phosphate (pH 7.2) and 5 mM tryptophan at room temperature. At the indicated times, aliquots (0.80 mL) were removed, mixed with 8 μL of 10 mM arachidonate (in ethanol), and incubated for a further 3 min at room temperature. Lipid products were extracted and analyzed by HPLC without derivatization to quantitate 11-HETE (open squares) and 15-HETE (filled circles), as described in Materials and Methods. The HETEs were identified from their retention times in reference to authentic standards and quantitated using an extinction coefficient of 23.3 mM^{-1} cm^{-1} at 235 nm (Graff et al., 1990); the values shown represent the amounts of HETEs produced in individual aliquots reacted with arachidonate.

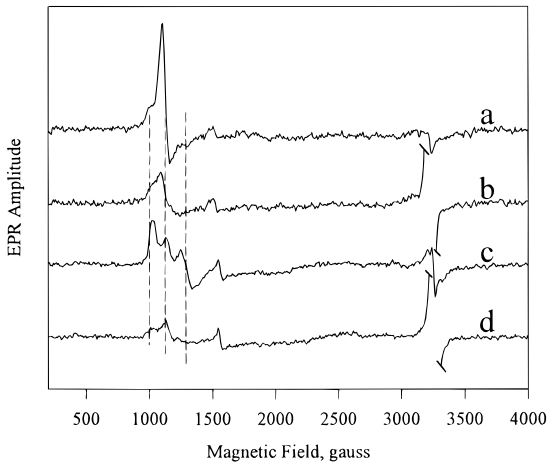


FIGURE 3: EPR spectra of human PGHS-2 and ovine PGHS-1 before and after reaction with ethyl hydroperoxide: (a) resting PGHS-2 (6.4 μM heme), (b) PGHS-2 after 6 s of reaction with 5 equiv of EtOOH at 0 °C, (c) resting PGHS-1 (17.8 μM heme), and (d) PGHS-1 after 6 s of reaction with 5 equiv of EtOOH at 0 °C. Spectra were recorded at a power level of 4 mW, a modulation amplitude of 10 G, a frequency of 9.27 GHz, and a temperature of 11 K. Corrections were made for a buffer blank.

decline in HETE production. Levels of synthesis of both HETEs peaked at about a 4.5-fold increase over the initial values (Figure 2), quite similar to the 4.8-fold activity increase observed from absorbance changes (Figure 1), supporting the validity of the more convenient spectrophotometric assay.

Characterization of Hydroperoxide-Induced Radicals in PGHS-2 and ASA-Treated PGHS-2. The EPR spectrum of resting PGHS-2 has several features near 1000 G attributable to high-spin ferric heme (Figure 3). The complex signal can most easily be interpreted in terms of an intense axial component with a peak at $g = 6.0$ and a zero crossing at $g = 5.8$, and a smaller component with low rhombicity which contributes the features at $g = 6.6$ and 5.1. The predominance of the axial component of high-spin heme in human PGHS-2 contrasts with the situation in ovine PGHS-1, where the major high-spin species is rhombic [Figure 3 and Kulmacz et al. (1987)]. The source of the trough at $g = 2.0$ in resting PGHS-2 is not known. There is a small derivative signal at $g = 4.1$ from adventitious iron. There is no indication of significant low-spin ferric heme in the resting PGHS-2. This is similar to PGHS-1, where low-spin ferric heme was observed only in more concentrated samples.

Addition of EtOOH to PGHS-2 resulted in loss of most of the high-spin heme signals and the appearance of an intense new radical signal near $g = 2$ (Figure 3). These overall peroxide-induced changes in the PGHS-2 EPR are similar to those observed for PGHS-1 (Figure 3). Both the axial and the rhombic high-spin components decreased upon reaction of either isoform with EtOOH, indicating that both axial and rhombic heme species were reactive.

For more detailed EPR observations of the effects of ASA treatment on the hydroperoxide-induced free radical, PGHS-2 holoenzyme was incubated with ASA for 0, 15, or 30 min before the enzyme was reisolated by gel filtration chromatography. The 15 min sample was designed to trap lipoxygenase activity near its maximum; the 30 min sample was included to examine changes associated with the secondary decrease in lipoxygenase activity (Figures 1 and 2). Each sample was reacted with EtOOH and then analyzed by EPR at liquid helium temperature (Figure 4A). EPR spectra obtained at liquid nitrogen temperature had the same line shapes as those in Figure 4A (data not shown).

For the control PGHS-2, reaction with hydroperoxide for 6 s at 0 °C produced a wide singlet species (29 G peak to trough) centered at $g = 2.0058$ (spectrum a in Figure 4A). This radical is similar to the radical observed with a microsomal preparation of PGHS-2 (Hsi et al., 1994) and slightly narrower than the wide singlet tyrosyl radical observed later in reactions of PGHS-1 with hydroperoxide (spectrum b in Figure 4B). Reasonable simulation of the PGHS-2 radical line shape was obtained using spectral parameters adjusted from those observed for the wide doublet tyrosyl radical in PGHS-1 (spectrum a′ in Figure 4A), indicating that the PGHS-2 EPR can be accounted for by a single radical species. Other manually mixed reactions of PGHS-2 with EtOOH which were quenched after as little as 4 s produced wide singlet EPR much like that shown in Figure 4A, with no indication of a wide doublet signal like that observed with PGHS-1 (spectrum a in Figure 4B). Formation of the wide singlet in PGHS-2 was very efficient (0.94 spin/heme) for the PGHS-2 sample in Figure 4A. Typical values for PGHS-1 are in the range of 0.5−0.7 spin/heme.

For PGHS-2 pretreated with ASA for 15 min, reaction with EtOOH resulted in formation of a wide singlet EPR signal with a line shape indistinguishable from that produced with control PGHS-2 (compare spectra a and b in Figure 4A). The half-saturation power value for the radical in ASA-treated PGHS-2 was 0.4 mW (at 98 K), essentially the same as that observed for the control (0.5 mW). Thus, the ASA pretreatment which maximized the lipoxygenase activity of PGHS-2 (Figures 1 and 2) did not alter perceptibly the structure of the tyrosyl radical produced by hydroperoxide. For PGHS-2 pretreated with ASA for 30 min, addition of EtOOH produced a singlet radical (spectrum c in Figure 4A) which was slightly narrower (27 G peak to trough) than those

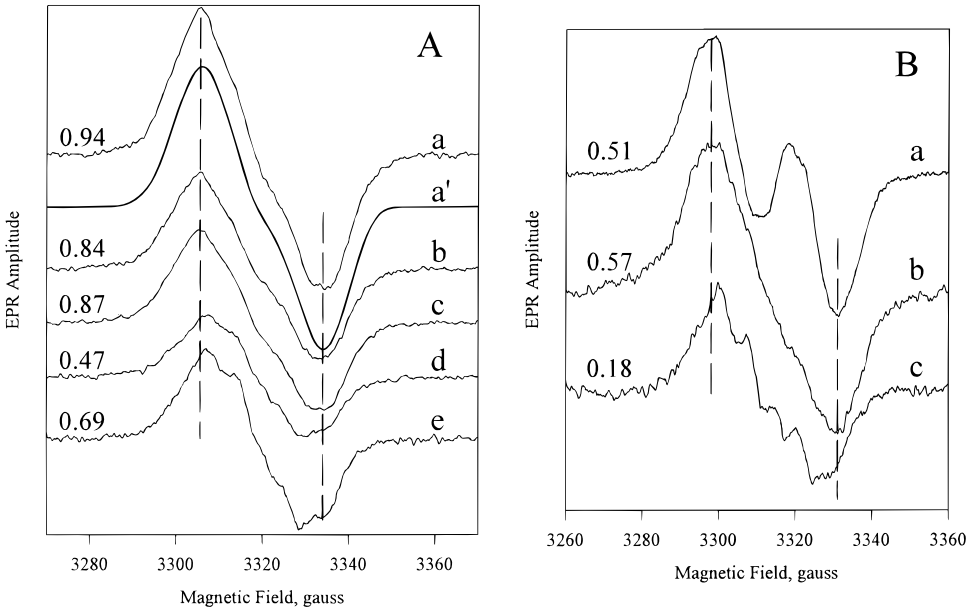


FIGURE 4: Detailed EPR spectra in the $g = 2$ region of PGHS isozymes after reaction with EtOOH. (A) Hydroperoxide-induced radicals in PGHS-2. PGHS-2 holoenzyme was treated with ASA as described in the legend to Figure 1. Aliquots were removed at 0, 15, and 30 min of incubation, and the protein was reisolated. The samples were reacted with 5 equiv of EtOOH at 0 °C for 6 s. Spectra were recorded at a power level of 10 μW, a modulation amplitude of 2 G, a frequency of 9.27 GHz, and a temperature of 11 K. Spectra are shown for (a) PGHS-2 (0 min with ASA), (b) PGHS-2 (15 min with ASA), (c) PGHS-2 (30 min with ASA), (d) PGHS-2 (1 equiv of nimesulide) and (e) PGHS-2 (30 min with ASA and then 1 equiv of nimesulide). Radical intensities (in spins per heme) are indicated at left. Spectrum a′ represents a simulated tyrosyl radical EPR. Details are described in Materials and Methods. (B) Hydroperoxide-induced radicals in PGHS-1. Samples were reacted with 10 equiv of EtOOH at −10 °C. Spectra were recorded at a power level of 10 μW, a modulation amplitude of 2 G, a frequency of 9.29 GHz, and a temperature of 12 K. Spectra are shown for (a) PGHS-1 after 15 s of reaction, (b) PGHS-1 after 20 min of reaction, and (c) PGHS-1 pretreated with ASA before reaction with EtOOH for 15 s. Radical intensities (in spins per heme) are indicated at left. Details are described in Materials and Methods and in Kulmacz et al. (1991).

observed with PGHS-2 treated for 0 or 15 min (spectra a and b in Figure 4A).

A separate sample of PGHS-2 was treated with a stoichiometric amount of nimesulide, an agent selective for PGHS-2 which produces time-dependent inhibition of the cyclooxygenase activity (Barnett et al., 1994); 76% inhibition was observed in the sample before EPR analysis. Upon subsequent reaction with EtOOH, the nimesulide-treated PGHS-2 produced a radical species which was much narrower (21 G peak to trough) than that from the sample pretreated with ASA (compare spectra a−c and d in Figure 4A). Addition of stoichiometric nimesulide to PGHS-2 previously pretreated with ASA for 30 min resulted in 84% inhibition of the residual oxygenase activity and an EtOOH-induced radical very similar to that observed in PGHS-2 with nimesulide alone (compare spectra d and e in Figure 4A). The radical in the sample treated with both ASA (30 min) and nimesulide was somewhat less intense (0.69 spin/heme) than that treated with ASA alone (0.84−0.87 spin/heme). The pronounced narrowing of the EPR line shape produced by nimesulide in PGHS-2 (spectra d and e in Figure 4A) was quite similar to that observed for ASA-treated PGHS-1 (spectrum c in Figure 4B).

The intensities of the hydroperoxide-induced radicals were followed as a function of the time of reaction (Figure 5). In each case, the intensity was highest at the first time examined (5−6 s) and declined afterward. The initial portion of the decline followed exponential kinetics, with a rate constant of about 0.01 s^{-1} (range of 0.008−0.012 s^{-1}) except for PGHS-2 treated with only nimesulide, where the rate constant was 0.023 s^{-1}. Thus, the radical in ASA-pretreated PGHS-2 decayed at a rate similar to that in control PGHS-2, whereas

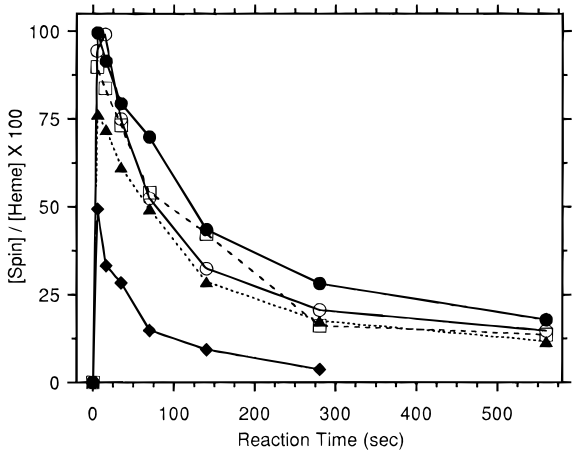


FIGURE 5: Intensities of PGHS-2 radicals during reaction with EtOOH. Each of the samples in Figure 4A was thawed to allow further reaction and the radical intensity determined at the indicated cumulative reaction times. Reaction kinetics are shown for PGHS-2 (open circles), PGHS-2 pretreated with ASA for 15 min (open squares), PGHS-2 pretreated with ASA for 30 min (filled circles), PGHS-2 pretreated with ASA for 30 min and then with 1 equiv of nimesulide (filled triangles), and PGHS-2 pretreated with 1 equiv of nimesulide (filled diamonds). Details are described in Materials and Methods.

the radical in nimesulide-treated PGHS-2 decayed considerably more quickly. It is interesting that treatment with ASA before nimesulide led to a more intense and more persistent radical than treatment with nimesulide alone (Figure 5), even though both samples appeared to have the same narrow radical species (Figure 4A). This suggests that acetylation of Ser516 may prevent some nimesulide-induced structural change in the protein. There was no indication of significant change in line shape for any of the samples as the reactions

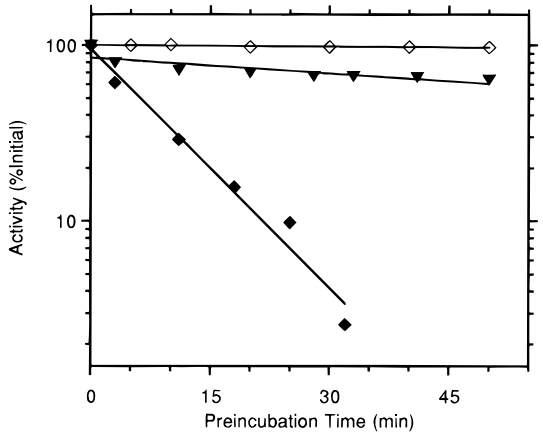


FIGURE 6: Inactivation of PGHS-2 cyclooxygenase activity by TNM. PGHS-2 holoenzyme (14 μM subunit) in 0.1 M potassium phosphate (pH 7.2)/0.1% Tween-20 was preincubated at room temperature with (filled symbols) or without (open symbols) 2.0 mM TNM. At the indicated times, aliquots were taken for assay of cyclooxygenase (diamonds) or peroxidase (triangles) activity under standard conditions. The initial activity was 31 units/mg for the cyclooxygenase and 15 units/mg for the peroxidase.

progressed (data not shown), indicating that the same radical species was present throughout.

Effects of TNM on Enzymatic Activities and EPR Spectrum of PGHS-2. Reaction with TNM was previously found to nitrate tyrosine residues in the PGHS-1 cyclooxygenase pocket, leading to selective loss of cyclooxygenase activity and a much narrower tyrosyl radical EPR (Kulmacz et al., 1990; Shimokawa et al., 1990). One of the active site tyrosine residues nitrated by TNM, Tyr385, is probably the location of the tyrosyl radical in native PGHS-1 (Tsai et al., 1994). The cyclooxygenase and peroxidase activities of recombinant PGHS-2 were examined as a function of the length of preincubation with TNM (Figure 6). The cyclooxygenase activity was rapidly inactivated, declining in an exponential fashion with a half-life of about 7 min. The peroxidase activity was more resistant to inactivation by TNM, with a half-life of about 100 min.

A sample of TNM-treated PGHS-2, retaining 88% of the control peroxidase activity but only 7% of the control cyclooxygenase activity, was reacted with EtOOH and the resulting radical analyzed by EPR (Figure 7). The radical in TNM-treated PGHS-2 was a narrow singlet (22 G peak to trough) almost identical with that observed for PGHS-2 inhibited with nimesulide (spectrum d in Figure 4A). The radical intensity in the TNM-treated enzyme was about half of that of the control (Figure 7), again similar to the observation with nimesulide-inhibited PGHS-2 (Figure 4A). Thus, inactivation of the PGHS-2 cyclooxygenase by TNM was accompanied by marked perturbation of the hydroperoxide-induced radical structure. The direct parallels between the effects of the tyrosine-modifying reagent TNM on PGHS-2 and on PGHS-1, where the radical in the native enzyme has been proved to reside on a tyrosine residue (Tsai et al., 1994), support the assignment of the hydroperoxide-induced 29 G radical in PGHS-2 to a tyrosyl radical.

Arachidonate Affinities in PGHS-2 and ASA-Treated PGHS-2. The dependencies of the oxygenase and lipoxygenase activities of native and ASA-treated PGHS-2 on arachidonate concentration were analyzed as described in Materials and Methods; the resulting K_m values are shown in Table 2. In native PGHS-2, the oxygenase activity had a much lower K_m for arachidonate (1 μM) than did the lipoxygenase activity (41 μM), implying that substrate binding was more effective for formation of the major, cyclooxygenase product, PGG_2, than for formation of the lipoxygenase side products, 11- and 15-HPETE. ASA treatment decreased the K_m of the lipoxygenase activity from 41 to 15 μM, indicating that acetylation of PGHS-2 increased the affinity for arachidonate as a substrate for HPETE formation. The lipoxygenase and oxygenase activities of ASA-treated PGHS-2 had indistinguishable K_m values, suggesting that the oxygenase activity is largely lipoxygenase activity or that surviving cyclooxygenase activity has impaired fatty acid binding.

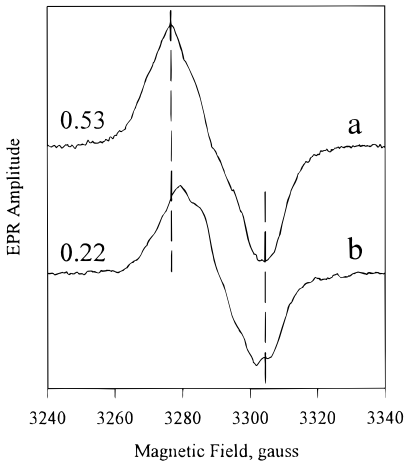


FIGURE 7: Effect of TNM pretreatment on EtOOH-induced radical in PGHS-2. PGHS-2 holoenzyme (10 μM subunit) was reacted with 2 mM TNM in 0.1 M potassium phosphate (pH 7.2)/0.1% Tween-20 for 22 min, before the enzyme was reisolated by gel filtration on a PD-10 column in the same buffer at 4 °C. After concentration by ultrafiltration on a YM-30 membrane, glycerol was added to 20%. A control sample was processed in the same manner, but without TNM. Samples were reacted with 5 equiv of EtOOH for 6 s at 0 °C. EPR spectra were recorded at a power level of 4 mW, a modulation amplitude of 3.2 G, a frequency of 9.22 GHz, and a temperature of 96 K. Spectra are shown for (a) control PGHS-2 (6.4 μM heme) and (b) TNM-treated PGHS-2 (5.6 μM heme); the amplitudes are normalized to the heme concentrations. Radical intensities (in spins per heme) are indicated at left.

Table 2: Oxygenase and Lipoxygenase K_m Values for PGHS-2 and ASA-Treated PGHS-2

	K_m (μM arachidonate)[a]	
	oxygenase[b]	lipoxygenase[c]
PGHS-2	0.93 ± 0.03	41 ± 3
ASA-treated PGHS-2[d]	13 ± 1	15 ± 2

[a] K_m and standard error values were from nonlinear least-squares fitting of the data at six to eight different arachidonate concentrations as described in Materials and Methods. [b] Measured by oxygen consumption. [c] Measured by increases in A_{235}. [d] PGHS-2 was preincubated with 0.8 mM ASA for 20 min and then reisolated by gel filtration as described in Materials and Methods.

Analysis of Lipoxygenase Product Stereochemistry for PGHS-2 and ASA-Treated PGHS-2. The stereochemistry of oxygen addition in the lipoxygenase reactions of PGHS-2 and ASA-treated PGHS-2 was determined by analysis of the 11- and 15-HETE formed as side products during reaction with arachidonate (Figure 8). For both native and ASA-treated PGHS-2, the derivatives of the 11- and 15-HETE reaction products comigrated with the *R* enantiomer of the corresponding racemic HETE standard. Essentially none of

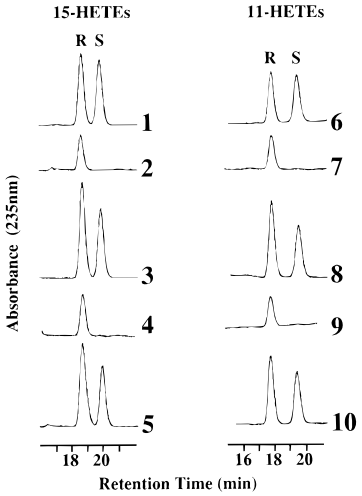


FIGURE 8: Stereochemical analysis of HETEs produced by PGHS-2 and ASA-treated PGHS-2. The holoenzyme was preincubated for 20 min with or without 0.8 mM ASA and then reacted with arachidonate. The resulting 11-HETE and 15-HETE were analyzed as described in Materials and Methods. Partial chromatograms are shown for the derivatives of the following: (1) 15(*RS*)-HETE standard, (2) 15-HETE from control PGHS-2, (3) coinjection of 15-HETE from control PGHS-2 and 15(*RS*)-HETE standard, (4) 15-HETE from ASA-treated PGHS-2, (5) coinjection of 15-HETE from ASA-treated PGHS-2 and 15(*RS*)-HETE standard, (6) 11-(*RS*)-HETE standard, (7) 11-HETE from control PGHS-2, (8) coinjection of 11-HETE from control PGHS-2 and 11(*RS*)-HETE standard, (9) 11-HETE from ASA-treated PGHS-2, and (10) coinjection of 11-HETE from ASA-treated PGHS-2 and 11(*RS*)-HETE standard. Chromatograms for the individual injections are normalized to the quantities used in the coinjections.

the *S* isomers was detected. Because reduction of HPETEs to HETEs by the synthase peroxidase activity retains the original configuration, these results demonstrated that, with or without acetylation of Ser516, lipoxygenation of arachidonic acid by PGHS-2 proceeded in a stereospecific manner to form 11(*R*)- and 15(*R*)-HPETE. Acetylation of the cyclooxygenase active site in PGHS-2 thus did not alter the stereochemistry of oxygen addition in the HPETE side products.

DISCUSSION

Lipoxygenase catalysis is a well-established side reaction of PGHS (Bailey et al., 1983; Setty et al., 1985; Hecker et al., 1987). The observed inhibition of lipoxygenase activity by anti-cyclooxygenase agents (Bailey et al., 1983; Setty et al., 1985) indicates that lipoxygenase catalysis occurs in the cyclooxygenase active site. In addition, the underlying chemical transformation in the lipoxygenase reaction, oxygen addition to a 1,4-pentadiene group in an unsaturated fatty acid, is shared by the cyclooxygenase reaction itself. It is thus reasonable to expect that the basic mechanism for the PGHS lipoxygenase reaction parallels the steps proposed for the initial part of the cyclooxygenase reaction, namely abstraction of a hydrogen atom from the fatty acid by an enzyme oxidant to generate a fatty acyl radical, followed by attack of the acyl radical on molecular oxygen to generate a hydroperoxide radical (Nugteren et al., 1966a). Examination of the PGHS lipoxygenase reaction has been hampered because it is only a minor reaction in the native enzyme (Hecker et al., 1987). The recent discovery that the lipoxygenase activity can be accentuated in PGHS-2, but not in PGHS-1, by acetylation with ASA has provided an opportunity for more detailed analysis of the lipoxygenase mechanism.

Acetylation of Ser516 in PGHS-2 was shown earlier to occur during incubation with ASA under mild conditions similar to those used in the present study (Lecomte et al., 1994; Wennogle et al., 1995). Little change in activity was observed when PGHS-2 was incubated in the absence of ASA (Figure 1). Thus, the changes in cyclooxygenase and lipoxygenase activities observed during incubation of PGHS-2 with ASA by others (Meade et al., 1993; Lecomte et al., 1994; O'Neill et al., 1994) and ourselves (Figure 1) are very likely to be consequences of acetylation of Ser516. Stimulation of PGHS-2 lipoxygenase activity by ASA treatment was found to be transient (Figures 1 and 2). The clearly biphasic effect of ASA preincubation on the lipoxygenase contrasted with the monotonic decrease in overall oxygenase activity, indicating that ASA has a second, inhibitory action on lipoxygenase catalysis. Such an inhibitory action is consistent with the observation that prolonged incubation with high levels of ASA completely inactivated the oxygenase activity in microsomal PGHS-2 (Laneuville et al., 1995). HETEs and their metabolites are known to be potent bioactive lipids, and it has been proposed that some of the actions of ASA in vivo might result from 15-HETE production by ASA-treated PGHS-2 (Meade et al., 1993; Claria & Serhan, 1995). The decrease in lipoxygenase activity seen with prolonged incubation of PGHS-2 with ASA (Figures 1 and 2) suggests that the ability of cellular PGHS-2 to generate HETEs, and to influence HETE-dependent events, may be attenuated with longer exposure to ASA.

The ASA pretreatment regimen which produced maximal PGHS-2 lipoxygenase activity did not lead to changes in the structure of the hydroperoxide-induced radical (Figures 1, 2, and 4A). On the other hand, nimesulide decreased the PGHS-2 lipoxygenase activity and led to alteration of the radical (Figure 4A). With PGHS-1, ASA inhibits all oxygenase activity and leads to a narrow singlet tyrosyl radical (spectrum c in Figure 4B). This correlation between retention of oxygenase activity and retention of the capacity to form the native radical species indicates that the same radical is used as the oxidant in PGHS-2 cyclooxygenase and lipoxygenase activities. Further, it supports the proposal (Karthein et al., 1988) that a tyrosyl radical is the key oxidant in catalysis by PGHS.

Reaction of PGHS-1 with hydroperoxide has been found to produce several types of EPR signals. The initial 34 G (peak to trough) doublet EPR signal observed in native PGHS-1, termed the wide doublet, is known to originate from a tyrosyl radical with a strained ring conformation (Karthein et al., 1988; Barry et al., 1990; Tsai et al., 1994). As reaction of PGHS-1 with hydroperoxide proceeds, the wide doublet EPR is replaced by a singlet characterized by a peak to trough width of about 32−34 G, termed the wide singlet (Kulmacz et al., 1990). PGHS-1 pretreated with ASA or many other anti-cyclooxygenase agents does not give a wide doublet or wide singlet EPR, but rather a narrow singlet characterized by distinct hyperfine features and a peak to trough width of about 26 G, assigned to a tyrosyl radical with a relaxed ring conformation (Barry et al., 1990; Kulmacz et al., 1991; Tsai et al., 1994). A narrow singlet with a peak to trough width of about 26 G is also observed with self-inactivated PGHS-1 (DeGray et al., 1992; Tsai et al., 1992); the EPR of this narrow singlet is not identical with the narrow singlet

observed in PGHS-1 treated with cyclooxygenase inhibitors (A.-L. Tsai and R. J. Kulmacz, unpublished observation). The wide singlet observed later during reaction of PGHS-1 with hydroperoxide can be accounted for as a summation of the wide doublet from native PGHS-1 and the narrow singlet from self-inactivated PGHS-1 (DeGray et al., 1992), but not the narrow singlet from indomethacin-treated PGHS-1 (A.-L. Tsai and R. J. Kulmacz, unpublished observation).

PGHS-2 produced much simpler EPR results during reaction with hydroperoxide than those from PGHS-1; the only EPR signal observed with native or ASA-treated PGHS-2 was a 29 G singlet (Figure 4A). This PGHS-2 singlet is distinctly narrower than the wide singlet tyrosyl radical EPR observed with PGHS-1 (Figure 4B), indicating some differences in structure between the PGHS-1 and PGHS-2 radicals. On the other hand, reaction of PGHS-2 with TNM led to a much narrower radical (Figure 7) and selective inhibition of the cyclooxygenase activity (Figure 6). These effects on PGHS-2 EPR and activity parallel those observed with PGHS-1, where TNM was found to nitrate Tyr385 in the cyclooxygenase pocket (Kulmacz et al., 1990; Shimokawa et al., 1990). In addition, mutagenesis of Tyr371 in PGHS-2 (corresponds to Tyr385 in PGHS-1) to a phenylalanine resulted in a loss of cyclooxygenase (but not peroxidase) activity and a pronounced narrowing of the hydroperoxide-induced radical EPR (D. Swinney and A.-L. Tsai, unpublished results). Similar effects were observed when Tyr385 in PGHS-1 was mutated to a phenylalanine (Tsai et al., 1994). Thus, chemical or mutagenic modification of critical tyrosine residue(s) in PGHS-2 altered the hydroperoxide-induced radical in PGHS-2 in the same fashion as that seen for the authentic tyrosyl radical in PGHS-1. Taken with the similar effects of cyclooxygenase inhibitors on the EPR of the two isoforms (discussed below) and the high level of amino acid conservation in their active sites, these results make it very likely that the hydroperoxide-induced radical in PGHS-2 is also a tyrosyl radical.

The PGHS-2 radical line shape can be accounted for by a single tyrosyl radical with a slightly different orientation of the methylene proton relative to the phenyl ring from that in the PGHS-1 wide doublet (spectra a and a′ in Figure 4A). This is in contrast with the PGHS-1 wide singlet, which is probably a summation of two tyrosyl radical species (DeGray et al., 1992). Thus, it appears that only one radical species was present during reaction of PGHS-2 or ASA-treated PGHS-2 with hydroperoxide. No wide doublet EPR was observed with PGHS-2, even at the shortest reaction times tested (Results). It may be that a wide doublet tyrosyl radical is not formed in PGHS-2, or that it progresses to the singlet species more rapidly than in PGHS-1, and thus was not trapped by the manual mixing method used. Treatment of PGHS-2 with nimesulide, which inhibited all oxygenase activity, led to a 22 G (peak to trough) singlet (Figure 4A); this signal can be accounted for by a tyrosyl radical with a relaxed ring conformation (simulation not shown). The singlet in nimesulide-treated PGHS-2 is clearly narrower than that in ASA-treated PGHS-1 (Figure 4). However, narrowing of the hydroperoxide-induced radical by oxygenase inhibitors is observed for both PGHS isoforms, implying that the narrow singlet species in the two isoforms do not serve as the oxidant for arachidonate in oxygenase catalysis (Tsai et al., 1995).

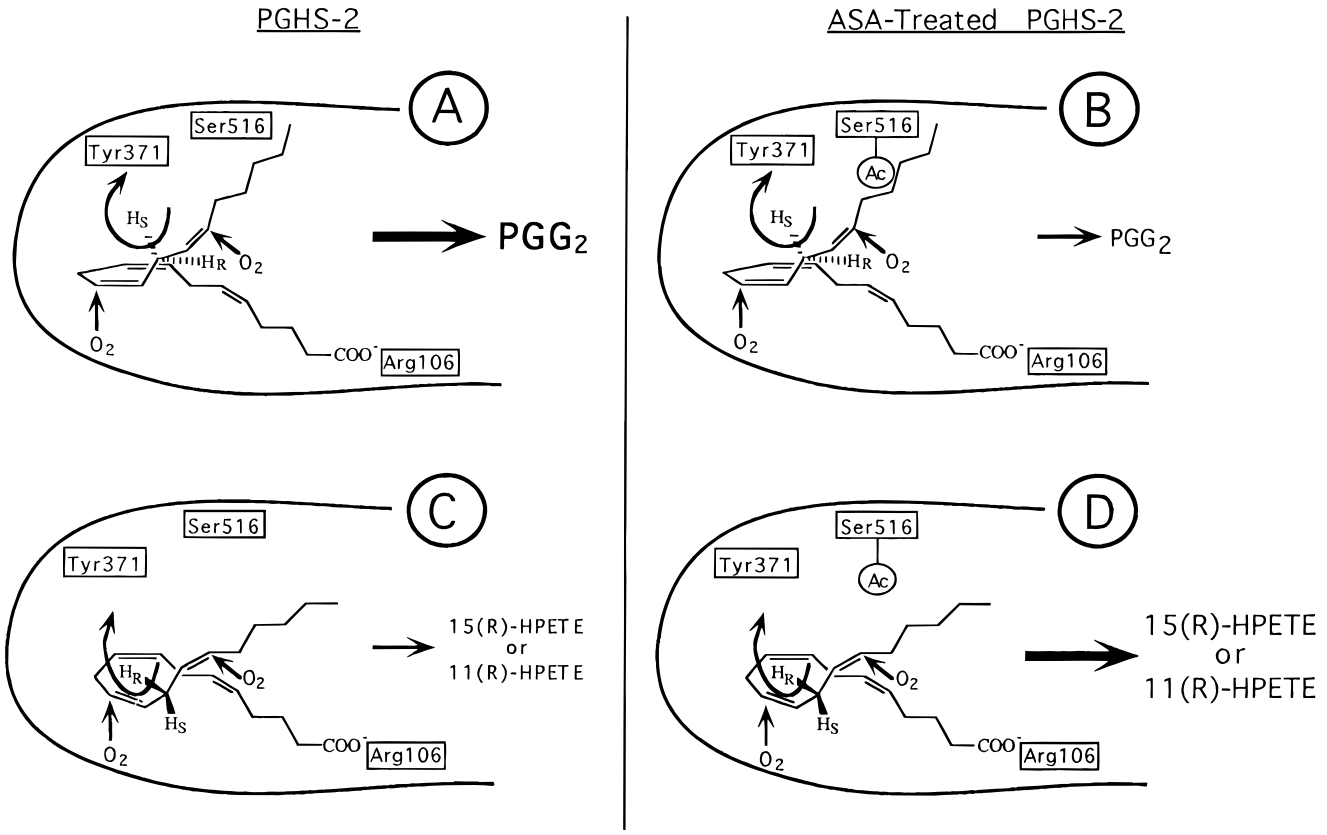
The hydroperoxide-induced radical seems to form in higher yield in PGHS-2 than in PGHS-1 (Figure 4), although there have been exceptions (Figure 7). Faster generation or greater stability of the radical in PGHS-2 may explain the higher yield. Given the proposed central role for the tyrosyl radical in cyclooxygenase catalysis (Karthein et al., 1988), more efficient accumulation of the radical in PGHS-2 is consistent with the recent observation that cyclooxygenase initiation requires a lower hydroperoxide level in PGHS-2 than in PGHS-1 (Capdevila et al., 1995; Kulmacz & Wang, 1995).

Utilization of a common enzyme oxidant for cyclooxygenase and lipoxygenase catalysis raises some interesting stereochemical issues because of the opposite configurations at C15 in PGH_2 (Nugteren et al., 1966b) and in the 15(*R*)-HETE produced by ASA-treated PGHS-2 (Figure 8; Holtzman et al., 1992; Lecomte et al., 1994; O'Neill et al., 1994). Antarafacial stereochemistry of hydrogen abstraction and oxygen addition is a well-established rule for fatty acid oxygenases (Yamamoto, 1991). The C13 (*pro-S*) hydrogen is removed during the cyclooxygenase reaction (Hamberg & Samuelsson, 1967); subsequent antarafacial oxygen addition would lead to a hydroperoxy radical at C11 with the *R* configuration and at C15 with the *S* configuration (Yamamoto, 1991). The formation of 15(*R*)-HETE by ASA-treated PGHS-2 led to the suggestion that acetylation of Ser516 altered the stereospecificity of oxygen addition (Lecomte et al., 1994). However, the earlier studies did not characterize the stereochemistry of 15-HETE produced by native, unacetylated PGHS-2. The present observation that 15(*R*)-HETE is produced by PGHS-2 both before and after treatment with ASA (Figure 8) demonstrates that the stereospecificity of oxygen addition at C15 is not disturbed by acetylation of Ser516.

To reconcile this retention of stereospecificity with the expected antarafacial orientation of hydrogen abstraction and oxygen addition in oxygenase reactions, we have devised a novel hypothesis regarding fatty acid binding by PGHS-2. In this interpretation, diagramed in Scheme 1, arachidonate can be bound in two distinct orientations in the cyclooxygenase channel. One of these orientations (A and B in Scheme 1) puts the C13 *pro-S* hydrogen of arachidonate in position to be abstracted by the enzyme oxidant (presumably a radical at Tyr371); antarafacial oxygen addition at both C11 and C15 forms the cyclooxygenase product, PGG_2. The other orientation (C and D in Scheme 1) has the methyl portion of arachidonate inverted by rotation of the C12−C13 bond so that the C13 *pro-R* hydrogen is abstracted by the enzyme oxidant; antarafacial oxygen addition at C11 or C15 leads to formation of the lipoxygenase products, 11-(*R*)- and 15(*R*)-HPETE. The alternative arachidonate conformation presumably also disrupts the cyclization steps required for formation of PGG_2, perhaps because of misalignment with crucial active site residues.

It is important to note that abstraction of the C13 *pro-S* hydrogen was established for PGE_1 formation but was not examined for the 15-hydroxy side product (Hamberg & Samuelsson, 1967). The proposed alternatives for arachidonate binding have no effect on the predicted configuration of 11-H(P)ETE because the orientation of C11 with respect to the abstracted hydrogen is undisturbed; antarafacial addition of oxygen produces the 11(*R*) isomer regardless of which hydrogen is abstracted from C13 (Scheme 1). This fits with the isolation of 11(*R*)-HETE from reactions with

Scheme 1: Hypothetical Mechanisms for Formation of PGG_2, 11(*R*)-HPETE, and 15(*R*)-HPETE in the Cyclooxygenase Active Site of Native PGHS-2 (Left Panel) and ASA-Treated PGHS-2 (Right Panel)[a]



[a] Arachidonic acid is proposed to be bound in two distinct conformations, one of which (A and B) positions the *pro-S* hydrogen on C13 near the enzyme oxidant (assumed to be a Tyr371 radical). Abstraction of the *pro-S* hydrogen and antarafacial addition of oxygen at C11 and C15 lead to formation of PGG_2. Alternatively (C and D), the fatty acid side chain is positioned so that the *pro-R* hydrogen is abstracted by the same enzyme oxidant, and antarafacial oxygen addition at either C11 or C15 produces 11(*R*)-HPETE or 15(*R*)-HPETE. In each case, the carboxyl group of the fatty acid interacts with Arg106. Major products are indicated by heavy arrows.

PGHS-2 and ASA-treated PGHS-2 (Figure 8). The present stereochemical results with native PGHS-2 are consistent with the observation that 11- and 15-HPETE produced by native PGHS-1 were predominantly the *R* stereoisomers (Hecker et al., 1987). In the present study, ASA treatment of pure PGHS-2 led to coordinate increases in 11- and 15-HETE (Figure 2), indicating little regioselectivity in oxygen addition after hydrogen abstraction from C13. This differs from results reported for microsomal PGHS-2 preparations, where 15-HETE production increased relative to that of 11-HETE after ASA treatment (O'Neill et al., 1994).

The proposal of distinct binding orientations for cyclooxygenase and lipoxygenase catalysis by PGHS-2 is supported by the observation of distinct K_m values for the two reactions by native PGHS-2 (Table 2). The decrease in the K_m value for the lipoxygenase activity after ASA treatment suggests that acetylation of Ser516 favors the "aberrant" arachidonate binding conformation (D in Scheme 1) over the "normal" conformation (B in Scheme 1). An earlier report found little difference in the K_m value for arachidonate after ASA treatment of PGHS-2 (Lecomte et al., 1994). However, the value obtained for ASA-treated enzyme may have reflected a considerable contribution from surviving cyclooxygenase activity because the reaction was monitored by oxygen consumption, and the ASA-treated PGHS-2 sample examined retained 75% of control activity. The spectrophotometric assay used in the present study has the benefit of being selective for lipoxygenase activity.

In summary, the present spectroscopic studies have shown that the same hydroperoxide-induced radical, presumably a tyrosyl radical, can be formed in native PGHS-2 and ASA-treated PGHS-2. Interaction of the radical with arachidonate bound in alternative conformations is proposed to account for observed differences in configuration at C15 in the cyclooxygenase and lipoxygenase products. These findings add to the accumulating evidence that a native tyrosyl radical plays a key role in oxygenase catalysis by both PGHS isoforms.

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