Detection of an Fe^{2+}−Protoporphyrin-IX Intermediate during Aspirin-Treated Prostaglandin H_2 Synthase II Catalysis of Arachidonic Acid to 15-HETE[†,‡]

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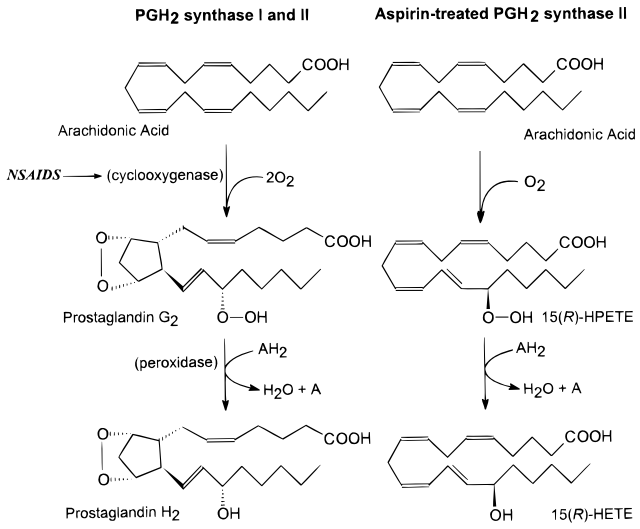
ABSTRACT: Spectral intermediates associated with the dioxygenase and peroxidase activities of prostaglandin H_2 (PGH_2) synthase I and II were monitored by stopped-flow spectrometry. During reactions of PGH_2 synthase I with arachidonic acid (AA) and ethyl hydrogen peroxide (EtOOH), compound I (Fe^{5+}; formally (protoporphyrin-IX)$\cdot^{+}Fe^{4+}$=O) and compound II (Fe^{4+}; formally (protoporphyrin-IX)Fe^{4+}=O) were detected. These intermediates were observed sooner with EtOOH (within 50 ms) than with AA (within 200 ms). Compound I and compound II were found to be kinetically competent with respect to AA-dependent O_2 uptake. These findings are consistent with a mechanism in which peroxidative cleavage precedes AA dioxygenation. During reactions with PGH_2 synthase II with AA, compound I and compound II were again observed within 200 ms and were kinetically competent to participate in dioxygenation. However, during reactions of PGH_2 synthase II with EtOOH, compound I and compound II were detected much later (after 10 s). These findings would be inconsistent with a mechanism in which peroxidative cleavage precedes AA dioxygenation. When aspirin-treated PGH_2 synthase II was reacted with EtOOH, a normal peroxidase cycle occurred with compound I and compound II formation occurring over 10 s. However, when aspirin-treated PGH_2 synthase II was reacted with AA, a unique spectral intermediate with λ_{max} at 446 nm was detected within 3 ms and was strikingly similar to ferrous (Fe^{2+}) protoporphyrin-IX. Aspirin-treated PGH_2 synthase II was found to produce 15-HETE, and the appearance of the Fe^{2+} intermediate (within 3 ms) indicated that it was kinetically competent to participate in the 15-dioxygenation event. The detection of this Fe^{2+} intermediate and the slow formation of compound I and compound II observed with EtOOH in PGH_2 synthase II suggest that peroxidative cleavage is not the initiating event in dioxygenation. Instead, it is proposed that the reduction of Fe^{3+} in heme to Fe^{2+} oxidizes a peroxide to yield an initiating peroxy radical. Since it is unlikely that 11- and 15-dioxygenation occurs via different mechanisms, our findings question mechanisms of catalysis in both PGH_2 synthases.

Prostaglandin H_2 (PGH_2) synthase (E.C. 1.14.99.1) catalyzes the 11- and 15-dioxygenation of arachidonic acid to yield PGG_2 (cyclooxygenase reaction) and the peroxidative cleavage of PGG_2 to yield PGH_2 (peroxidase reaction) (Scheme 1) (Hamburg & Samuelsson, 1967, 1973). These reactions constitute the first steps in the biosynthesis of prostaglandins which mediate symptoms of inflammation. PGH_2 synthase is the primary target for nonsteroidal anti-inflammatory drugs (NSAIDs) (Vane, 1971; Vane & Botting, 1987). These drugs inhibit the cyclooxygenase reaction only, blocking the formation of the primary prostaglandins that mediate symptoms of inflammation (Vane, 1974; Humes et al., 1981).

Scheme 1: Reactions Catalyzed by PGH_2 Synthase I and II and by Aspirin-Treated PGH_2 Synthase II



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[1] Abbreviations: AA, arachidonic acid; PGH_2, prostaglandin H_2 synthase (E.C. 1.14.99.1); 15-HETE, 15-hydroxy-(*Z,Z,Z,E*)-5,8,11,13-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-(*Z,Z,Z,E*)-5,8,11,-13-eicosatetraenoic acid; GSH, glutathione; RP-HPLC, reverse-phase high-performance liquid chromatography.

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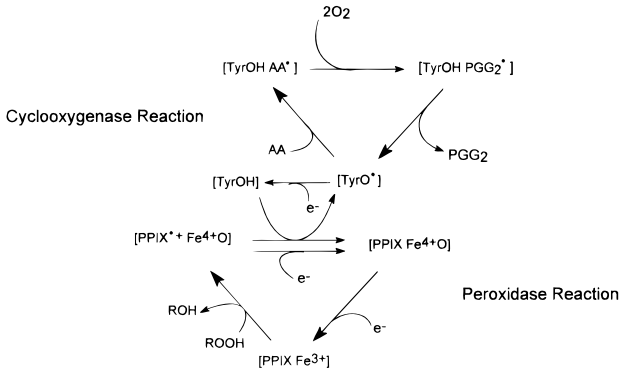
Scheme 2: Proposed Mechanism for PGH_2 Synthase Catalysis (Karthein et al., 1988; Dietz et al., 1988; Ruf et al., 1993)[a]



[a] Key: PPIX = protoporphyrin-IX; ROOH = hydroperoxide; TyrOH = tyrosine 385; AA = arachidonic acid; $PPIX^{\bullet+}Fe^{4+}O$ = compound I; $PPIXFe^{4+}O$ = compound II.

PGH_2 synthase functions as a homodimer with a monomer M_r = 72 kDa. Each monomer contains heme, a Fe^{3+}–protoporphyrin-IX, which is essential for both the cyclooxygenase and peroxidase activities (Ohki et al., 1979). It has been proposed that PGH_2 synthase activates arachidonic acid for O_2 insertion by abstracting the 13-pro(*S*)-hydrogen to form a carbon-centered radical and that the oxidant is a tyrosyl radical (Karthein et al., 1988; Mason et al., 1980; Tsai et al., 1994, 1995). This tyrosyl radical is generated by a heterolytic cleavage of a hydroperoxide with a concomitant change in the oxidation state of the iron in heme from Fe^{3+} to Fe^{5+}. A proposed mechanism of catalysis predicts an obligatory requirement for peroxide bond cleavage via formation of the equivalent of compound I (Fe^{5+}; formally (protoporphyrin-IX)$^{\bullet+}Fe^{4+}$=O) and compound II (Fe^{4+}; formally (protoporphyrin-IX)Fe^{4+}=O) of horseradish peroxidase (Scheme 2) (Karthein et al., 1988; Dietz et al., 1988; Ruf et al., 1993). According to the mechanism proposed by Ruf and colleagues (Karthein et al., 1988; Dietz et al., 1988; Ruf et al., 1993), compound I gives rise to the tyrosyl radical. Compound I and compound II have been observed during reactions of PGH_2 synthase with either arachidonic acid or peroxidase substrates (Nastainczyk et al., 1984; Lambeir et al., 1985).

There are two isoforms of the PGH_2 synthase, the constituitively expressed form, PGH_2 synthase I, and the inducible form, PGH_2 synthase II. PGH_2 synthase I was initially purified (Miyamoto et al., 1976; Hemler et al., 1976; Okhi et al., 1979) and cloned from ram seminal vesicles (DeWitt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988) and is constituitively expressed in most tissues (Simmons et al., 1991) and in blood platelets (Funk et al., 1991). PGH_2 synthase II is expressed only after induction during cell activation by cytokines, growth factors, tumor promoters, or mediators of inflammation (Kujubu et al., 1991; Xie et al., 1991; O'Banion et al., 1992; Hla, T., & Nielson, K., 1992). PGH_2 synthase II has been linked to inflammatory cell types and tissues and is believed to be the principal target for the anti-inflammatory activity of the NSAIDs (Fu et al., 1990; Masferrer et al., 1994; Sano et al., 1992).

In this study, the formation of compound I and compound II in PGH_2 synthase I and II was followed by stopped-flow spectrometry using arachidonic acid and ethyl hydroperoxide (EtOOH) as substrates. The formation of compound I and compound II during reactions catalyzed by PGH_2 synthase I and II were correlated with arachidonic acid-dependent oxygen consumption monitored by computerized Clark-style oxygen electrodes. Our studies show that PGH_2 synthase I and II produce compound I and compound II with peroxidase substrates on very different time courses. In PGH_2 synthase II, the formation of these intermediates does not permit peroxides to initiate arachidonic acid dioxygenation through higher oxidation states of iron.

The two isoforms of PGH_2 synthase are pharmacologically distinct (Holtzman et al., 1992; Meade et al., 1993). Previous studies have indicated that aspirin-treated PGH_2 synthase II produces 15(*R*)-hydroxyeicosatetraenoic acid [15(*R*)-HETE] from arachidonic acid (Holtzman et al., 1992; Mancini et al., 1994; O'Neill et al., 1994). The spectral intermediates associated with this activity are also reported. Our studies show that while aspirin-treated PGH_2 synthase II catalyzes a normal peroxidase cycle, employing compound I and compound II, dioxygenation of arachidonic acid is preceded by a ferrous (Fe^{2+}) form of the enzyme. This work represents the first study of transient spectral intermediates involved in PGH_2 synthase II catalysis and questions the existing mechanisms for arachidonic acid dioxygenation catalyzed by both synthases.

EXPERIMENTAL PROCEDURES

Materials. TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine), DDC (diethyldithiocarbamic acid), sodium dithionite, phenol, aspirin, and hematin were purchased from Sigma (St. Louis, MO). Arachidonic acid was purchased from Nu Chek Prep, Inc. (Elysian, MN). EtOOH (ethyl hydrogen peroxide) was purchased as a 5% solution in H_2O from Polysciences (Warrington, PA). PPHP (5-phenyl-4-pentenylhydroperoxide) was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). [1-^{14}C]Arachidonic acid (55.0 mCi/mmol) was purchased from NEN/Dupont (Boston, MA). 15(±)-HETE was purchased from Cayman Chemicals (Ann Arbor, MI).

Enzymes. PGH_2 synthase I was purified to homogeneity from ram seminal vesicles as described previously (Marnett et al., 1984). PGH_2 synthase I was reconstituted with 10-fold excess heme to form holo-PGH_2 synthase I. Free heme was removed by centrifugation in an Amicon Centriprep-10 concentrator (Beverly, MA). PGH_2 synthase II was purified to homogeneity from *Baculovirus*-infected Sf21 insect cells as described recently (George et al., 1996). PGH_2 synthase II was reconstituted in the manner described.

Cyclooxygenase Assay. The bis-dioxygenation of arachidonic acid to yield prostaglandin G_2 (PGG_2) and the dioxygenation of arachidonic acid to yield 15-hydroperoxyeicosatetraenoic acid [15(*R*)-HPETE] was followed by measuring oxygen consumption using a computerized Clark-style oxygen microelectrode (Instech; Plymouth Meeting, PA). These electrodes permit acquisition of 1000 data points/min, and the dead time of the electrode is 60 ms. The standard assay chamber (600 μL) contained 100 mM Tris-HCl (pH 8.0), 1 mM phenol, 100 μM hematin, and 100 μM arachidonic acid. The reactions were initiated by the addition of enzyme. By using this procedure, PGH_2 synthase I had a specific activity of 30 μmol of oxygen consumed/min/mg of enzyme and PGH_2 synthase II had a specific activity of 10 μmol of oxygen consumed/min/mg of enzyme.

Peroxidase Assay. The two-electron reduction of H_2O_2 using TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine) as the reducing cosubstrate was measured spectrophotometrically (Kulmacz & Lands, 1987; Kulmacz, 1989). The reaction system (1.0 mL) contained 20 μg of PGH_2 synthase I or II, 100 mM Tris-HCl (pH 8.0), 100 μM hematin, 80 μM TMPD, and 200 μM hydrogen peroxide. The reaction was initiated by the addition of TMPD. The formation of *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediimine ($E_{611} = 13\,500$ M^{-1} cm^{-1}) was complete within 60 s. By using this procedure, PGH_2 synthase I had a specific activity of 20 μmol of TMPD reduced/min/mg of enzyme and PGH_2 synthase II had a specific activity of 12 μmol of TMPD reduced/min/mg of enzyme.

Aspirin-Treated PGH_2 Synthase II. To generate aspirin-treated PGH_2 synthase II, holo-PGH_2 synthase II (4.0 μM) was incubated with 3 mM aspirin in 500 μL of 100 mM Tris-HCl, pH 8.0, 5% (v/v) EtOH at 25 °C for 6 h. Aliquots (10 μL) were removed over time and the arachidonic acid-dependent oxygen uptake was measured. After 5 h, there was no further decline in this activity. At this time point, the rate of oxygen uptake was 50% of the initial cyclooxygenase activity. The peroxidase activity of the aspirin-treated PGH_2 synthase II was reassayed after the aspirin treatment and was found to be identical with untreated enzyme. Aspirin-treated PGH_2 synthase II was prepared fresh for each experiment. In some experiments, free aspirin was removed from the aspirin-treated enzyme by centrifugation in an Amicon Centriprep-10 concentrator. The dioxygenase and peroxidase activities of the aspirin-treated enzyme were similar irrespective of whether free aspirin was removed.

15-HETE Production by Aspirin-Treated PGH_2 Synthase II. Aspirin-treated synthase (2.0 μM) was incubated with 36 μM [1-^{14}C]arachidonic acid (1.0 μCi) in 0.5 mL buffer containing 100 mM Tris-HCl, pH 8.0, 1 mM phenol for 10 min at 25 °C. Identical incubations were performed with untreated PGH_2 synthase II. The reaction mixtures were extracted with 5 vol of ethyl acetate/HOAc (99:1, v/v) and 2 vol of 0.15 M NaCl and extracted twice with 2 vol of CH_3CN/HOAc (99:1, v/v). The organic phases were pooled and dried under argon and re-dissolved in 2.0 mL CH_3CN/H_2O/HOAc (40:59:1, v/v). The reaction products were resolved by RP-HPLC on a 5 μm Spherisorb ODS2 C_{18} column (4.6 × 250 mm, PhaseSep, Franklin, MA) utilizing mixtures of solvent A [H_2O/HOAc (99:1, v/v)] and solvent B [CH_3CN/HOAc (99:1, v/v)]). Initial conditions were 60% A/40% B. Fifteen minutes after injection, the solvent composition was changed linearly to 30% A/70% B over 30 min. After a 5 min isocratic period, the composition of the solvent mixture was changed linearly to 100% B over 15 min and maintained at 100% B for an additional 5 min. The flow rate was 1.0 mL/min. The fractions were collected and aliquots counted on a scintillation counter for the detection of ^{14}C-radioactivity.

Stopped-Flow Spectrometry. The stopped-flow experiments were performed on a computerized stopped-flow apparatus with a rapid scanning monochrometer RSM-1000 (On-Line Instrument System, Inc., GA). Absorbance spectra through the Soret band (350−550 nm) were continuously scanned once every millisecond. Kinetic traces of changes in absorbance at any wavelength within the scanned region can be extracted from the primary data. The dead time of the stopped-flow apparatus was 3 ms. The reaction system (150 μL) contained 100 mM Tris-HCl (pH 8.0) and 13 μM diethyldithiocarbamic acid. Since the PGH_2 synthase was prepared as an apo-enzyme, hematin was added to achieve a 1.2 ratio of hematin:PGH_2 synthase in the reaction chamber. One syringe of the stopped-flow apparatus contained 1.6−2.2 μM enzyme. The other syringe contained the substrate.

Detection of Compound I and Compound II during Reactions of PGH_2 Synthase I and II. PGH_2 synthase I or II (0.8−1.1 μM) was reacted with 200 μM ethyl hydrogen peroxide, 50 μM PPHP, or 50 μM arachidonic acid, and the formation of compound I and compound II was followed on the stopped-flow spectrometer. To follow the formation of compound I, a kinetic trace of the change in absorbance at 411 nm was monitored. To follow the formation of compound II, individual time-resolved spectra were examined for the formation of a Soret band at 420 nm and the kinetic trace at 411 nm was examined to determine when compound I formation was complete.

Detection of Spectral Intermediates during Reactions of Aspirin-Treated PGH_2 Synthase II. Aspirin-treated PGH_2 synthase II (0.8 μM) was reacted with 200 μM ethyl hydrogen peroxide, 50 μM PPHP, or 50 μM arachidonic acid, and the formation of spectral intermediates was followed on the stopped-flow spectrometer. The formation of compound I and compound II were followed as described above. To follow the formation of the Fe^{2+} intermediate, a kinetic trace of the change in absorbance at 446 nm was measured.

RESULTS AND DISCUSSION

Formation of Compound I and Compound II during Reactions Catalyzed by PGH_2 Synthase I. To verify that compound I (Fe^{5+}) and compound II (Fe^{4+}) form during arachidonic acid dioxygenation, PGH_2 synthase I was incubated with this fatty acid and the formation of higher oxidation states of iron were monitored by stopped-flow spectrometry. Changes in oxygen consumption were measured concurrently by using computerized oxygen electrodes. In these experiments, compound I (falling 411 nm) was detected after 100 ms and compound II (Soret-band at 420 nm) was detected after 200 ms (Figure 1, panels C and D). Also, oxygen consumption began within 200 ms after the addition of arachidonic acid (Figure 1, panels E and F). When ethyl hydrogen peroxide was substituted as substrate, compound I was detected within 50 ms and compound II was detected after 100 ms (Figure 1, panels A and B).

With arachidonic acid, compound I and compound II were observed concurrently with arachidonic acid-dependent oxygen consumption. This suggests that compound I and compound II are kinetically competent species that can participate in the dioxygenation and/or peroxidase reactions. In this study, compound I and compound II were observed sooner when ethyl hydrogen peroxide was substituted for arachidonic acid. These findings are in agreement with previous studies on the formation of compound I and compound II by PGH_2 synthase I (Lambeir et al., 1985; Dietz et al., 1988). It has been suggested that compound I and compound II are formed when PGH_2 synthase I reacts with low levels of contaminating species of hydroperoxides (initiator peroxide) present in arachidonic acid (Marshall at al., 1987). Therefore, additional time is required for the formation and accumulation of compound I and compound II with this substrate. This would account for the gradual

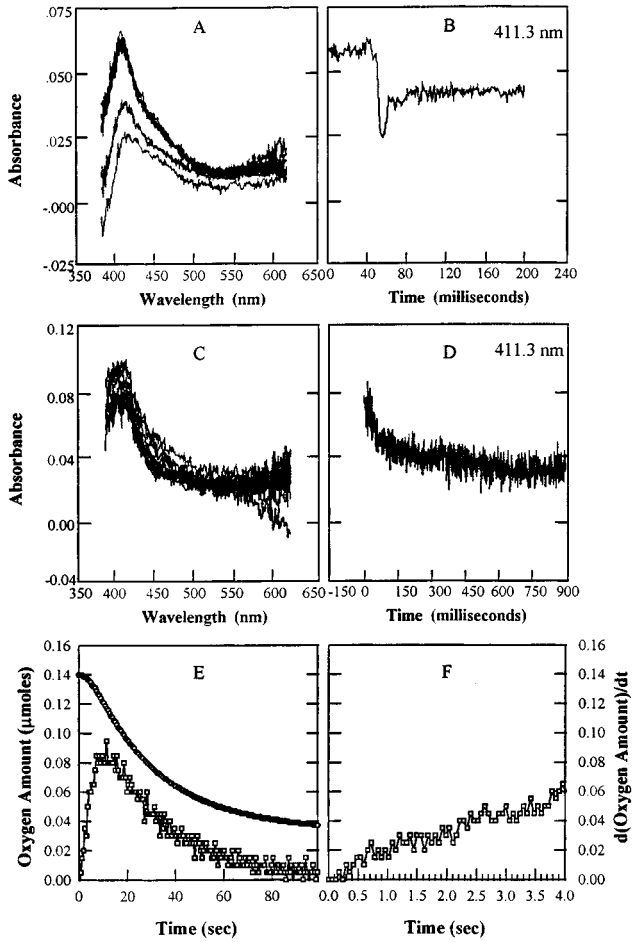


FIGURE 1: Time-resolved spectra showing the formation of compound I and compound II and dioxygenation of arachidonic acid catalyzed by PGH_2 synthase I. PGH_2 synthase I (1.0 μM) was reacted with 200 μM ethyl hydrogen peroxide, and the formation of compound I and compound II was followed on the stopped-flow spectrometer. (A) Absorbance spectra of the Soret band region for PGH_2 synthase I and (B) kinetic trace of changes in absorbance at 411 nm. PGH_2 synthase I (1.0 μM) was reacted with 50 μM arachidonic acid, and the formation of compound I and compound II was followed on the stopped-flow spectrometer. (C) Absorbance spectra of the Soret band region for PGH_2 synthase I and (D) kinetic trace of changes in absorbance at 411 nm. In parallel experiments, arachidonic acid-dependent oxygen uptake was measured. Oxygen consumption was monitored by computerized Clark-styled oxygen electrodes. (E) Change in the amount of oxygen in the chamber over time, (○−○), and the first derivative of the change in amount of oxygen in the chamber, (□−□). (F) the first derivative of the change in amount of oxygen in the chamber in the first 4 s (□−□).

buildup of compound I observed over 500 ms and its continuous conversion to compound II observed in the incubations with arachidonic acid.

Formation of Compound I and Compound II during Reactions Catalyzed by PGH_2 Synthase II. To determine whether compound I and compound II form during reactions catalyzed by PGH_2 synthase II, recombinant human enzyme expressed and purified from *Baculovirus*-infected Sf21 insect cells was incubated with either arachidonic acid or ethyl hydrogen peroxide. Compound I and compound II were again monitored by stopped-flow spectrometry and arachidonic acid-dependent oxygen uptake was measured with oxygen electrodes. When arachidonic acid was used as the substrate, compound I was detected after 100 ms and compound II was detected after 200 ms (Figure 2, panels C and D). Oxygen consumption was found to occur within 200 ms after the addition of arachidonic acid (Figure 2, panels E and F). Therefore, compound I and compound II were observed concurrently with arachidonic acid-dependent oxygen consumption. When ethyl hydrogen peroxide was used as the substrate in the stopped-flow spectrometer, compound I was detected only after 4 s and compound II was detected after 10 s (Figure 2, panels A and B). Thus, with ethyl hydrogen peroxide, compound I and compound II were observed much later than the initiation of arachidonic acid-dependent oxygen consumption. When PPHP was used as the substrate in the stopped-flow spectrometer, the rate of formation of compound I and compound II was similar to that observed when ethyl hydrogen peroxide was used as the substrate (Figure 3, panels A and B). Thus, irrespective of the peroxidase substrate, compound I and compound II form much more slowly with hydroperoxides than with

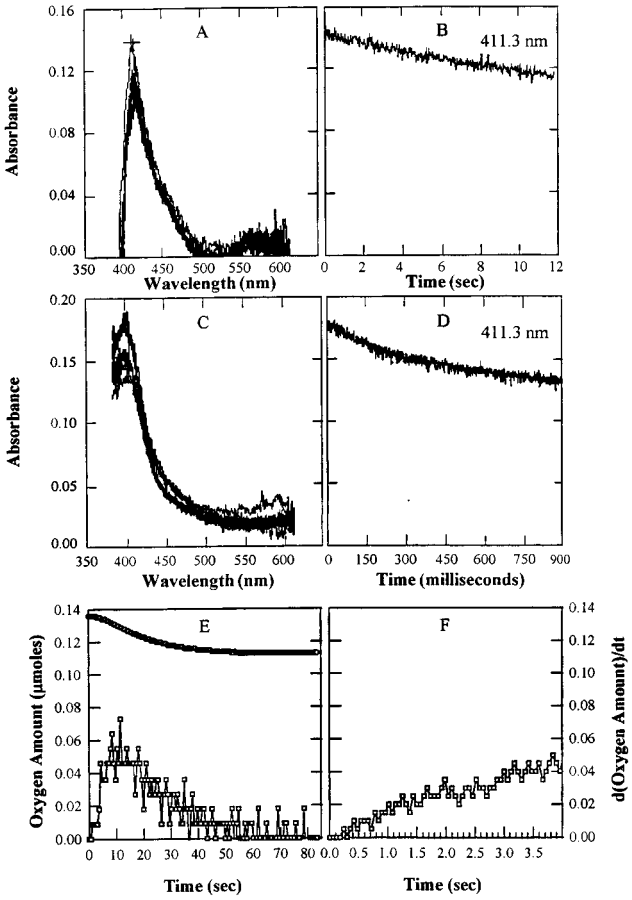


FIGURE 2: Time-resolved spectra showing the formation of compound I and compound II and dioxygenation of arachidonic acid catalyzed by PGH_2 synthase II. PGH_2 synthase II (1.1 μM) was reacted with 200 μM ethyl hydrogen peroxide, and the formation of compound I and compound II was followed on the stopped-flow spectrometer. (A) Absorbance spectra of the Soret band region for PGH_2 synthase II and (B) kinetic trace of changes in absorbance at 411 nm. PGH_2 synthase II (1.1 μM) was reacted with 50 μM arachidonic acid, and the formation of compound I and compound II was followed on the stopped-flow spectrometer. (C) Absorbance spectra of the Soret band region for PGH_2 synthase II and (D) kinetic trace of changes in absorbance at 411 nm. In parallel experiments, arachidonic acid-dependent oxygen uptake was measured. Oxygen consumption was monitored by computerized Clark-styled oxygen electrodes. (E) Change in the amount of oxygen in the chamber over time, (○−○) and the first derivative of the change in amount of oxygen in the chamber (□−□). (F) The first derivative of the change in amount of oxygen in the chamber in the first 4 s (□−□).

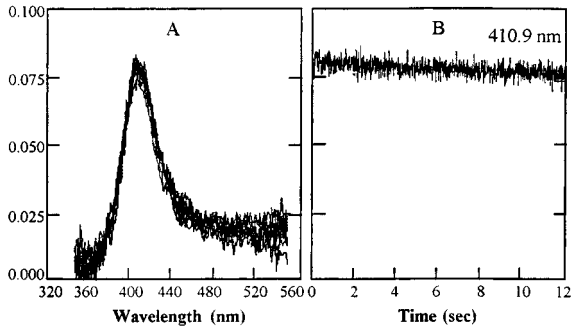


FIGURE 3: Time-resolved spectra showing the formation of compound I and compound II during PGH_2 synthase II reaction with PPHP. PGH_2 synthase II (1.1 μM) was reacted with acid 50 μM PPHP and the formation of compound I and compound II was followed on the stopped-flow spectrometer. (A) Absorbance spectra of the Soret band region for PGH_2 synthase II and (B) kinetic trace of changes in absorbance at 411 nm.

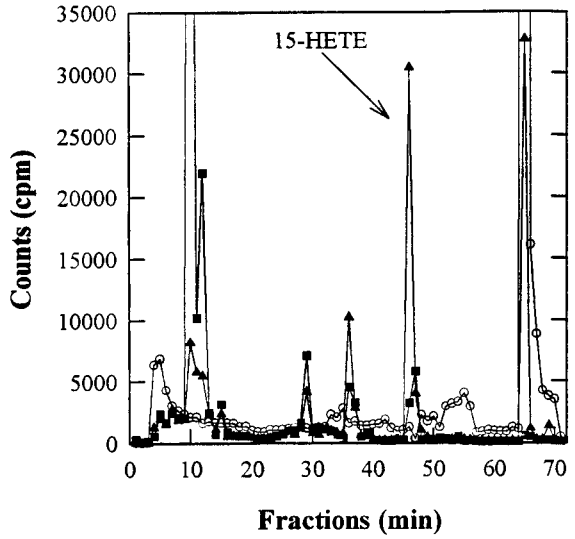


FIGURE 4: Product-profile of PGH_2 synthase II and aspirin-treated PGH_2 synthase II using arachidonic acid as substrate. The following reaction mixtures were extracted and subjected to radiochromatography by RP-HPLC as described in the Experimental Procedures. 1-[^{14}C]AA substrate (18 nmol) after incubation in buffer alone (100 mM Tris-HCl pH 8.0, 1 mM phenol), (○−○). 1-[^{14}C]AA substrate (36 nmol) after incubation in buffer containing 4.0 μM holo-PGH_2 synthase II (■−■). 1-[^{14}C]AA substrate (36 nmol) after incubation in buffer containing 4.0 μM aspirin-treated PGH_2 synthase II (▲−▲).

arachidonic acid.

Previous studies have suggested that compound I and compound II are formed when PGH_2 synthase I catalyzes the peroxidative cleavage of initiator hydroperoxides resulting in the formation of Fe^{5+} and Fe^{4+} (Nastainczyk et al., 1984; Lambeir et al., 1985; Marshall at al., 1987). This then leads to the generation of a tyrosyl radical which initiates the cyclooxygenase reaction. However, we detected compound I and compound II during the PGH_2 synthase II reaction with arachidonic acid before they were detected during reactions with ethyl hydrogen peroxide or PPHP. Therefore, it is unlikely that compound I and compound II could be derived from a contaminating hydroperoxide species. This would suggest that contaminating hydroperoxides present in arachidonic acid do not initiate the cyclooxygenase reaction of PGH_2 synthase II by undergoing heterolytic bond cleavage catalyzed by the peroxidase activity. This observation raises questions about the catalytic mechanism of PGH_2 synthase II and the necessity of the peroxidase reaction to initiate the cyclooxygenase reaction.

To ensure that compound I and compound II were not being formed with ethyl hydrogen peroxide before they could be detected in the stopped-flow spectrometer, the reaction rate was decreased using lower concentrations of substrate. When 1 and 50 μM ethyl hydrogen peroxide were incubated with holo-PGH_2 synthase II, compound I and compound II were detected after 4 and 10 s, respectively. This was consistent with the time course measured using 200 μM ethyl hydrogen peroxide. It should be noted that the dead time of the spectrometer is only 3 ms.

Aspirin-Treated PGH_2 Synthase II Produces 15-HETE. Previous studies have shown that aspirin-treated PGH_2 synthase II catalyzes the 15-dioxygenation of arachidonic acid leading to the formation of 15(*R*)-HPETE (Holtzman et al., 1992; Mancini et al., 1994; O'Neill et al., 1994), which is then a substrate for the peroxidase activity. To study this 15-dioxygenase reaction, holo-PGH_2 synthase II was incubated with aspirin for 5 h. Aliquots of the aspirin-treated enzyme were assayed for arachidonic acid-dependent oxygen consumption in the oxygen electrode chamber over time. After 5 h, it was found that there was no further decrease in the rate of oxygen consumption observed in the presence of arachidonic acid and that the aspirin-treated enzyme retained about 50% of the initial rate of oxygen consumption observed in untreated enzyme (Figures 2F and 5F). The peroxidase activity was also monitored throughout the aspirin-treatment and was found to be unaffected. At the end of the incubation period (5 h), the enzyme was used in the stopped-flow experiments.

The product-profile of aspirin-treated PGH_2 synthase II was also monitored to confirm that the enzyme catalyzes the formation of 15(*R*)-HETE from arachidonic acid. 1-[^{14}C]-Arachidonic acid was incubated with aspirin-treated PGH_2 synthase II, and the reaction mixture was extracted with ethyl acetate and analyzed by RP-HPLC. A major radioactive peak with a retention time corresponding to that observed for authentic synthetic 15(±)-HETE was detected (Figure 4). The absence of prostaglandins in the product-profile indicated that the aspirin treatment had converted all the enzyme into a 15(*R*)-lipoxygenase.

Formation of Compound I and Compound II during the Reaction of Aspirin-Treated PGH_2 Synthase II with Ethyl Hydrogen Peroxide. To determine whether compound I and compound II form during the reactions catalyzed by aspirin-treated PGH_2 synthase II, the enzyme was incubated with ethyl hydrogen peroxide and changes in the Soret band absorbance were monitored by stopped-flow spectrometry. Compound I was observed within 4 s, and compound II occurred after 10 s (Figure 5, panels A and B). When PPHP was substituted, the rate of formation of compound I and compound II were similar to that observed with ethyl hydrogen peroxide as the substrate. These observations were essentially identical to those seen for untreated PGH_2 synthase II. This would indicate that the peroxidase activity of PGH_2 synthase II is unaffected by aspirin treatment. In addition, the peroxidase activity was assayed using H_2O_2 and TMPD as reducing cosubstrate and was found to remain unchanged, versus untreated enzyme.

Formation of a Unique Spectral Intermediate during the 15-Dioxygenation of Arachidonic Acid Catalyzed by Aspirin-Treated PGH_2 Synthase II. To determine whether aspirin-

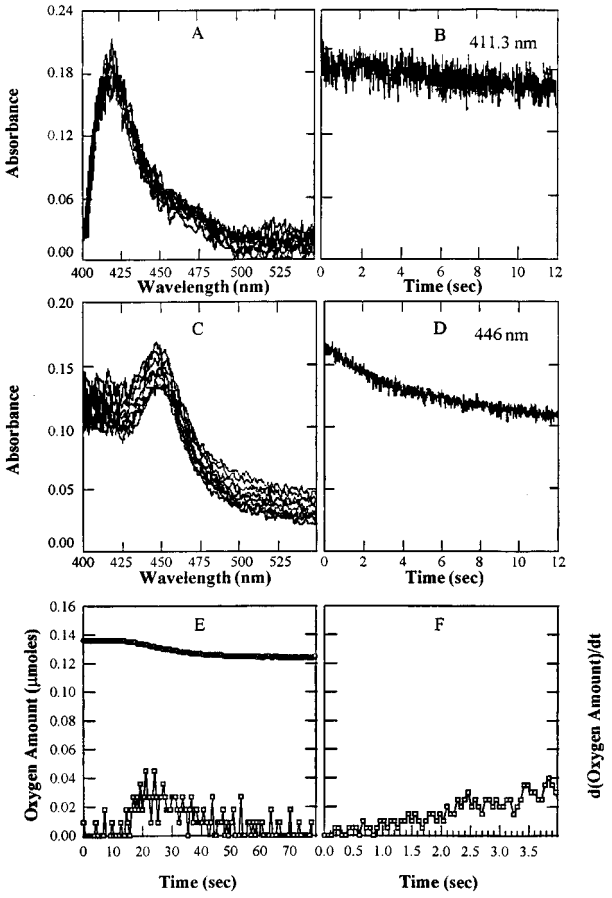


FIGURE 5: Time-resolved spectra showing the formation of compound I and compound II, a Fe^{2+} intermediate, and dioxygenation of arachidonic acid catalyzed by aspirin-treated PGH_2 synthase II. Aspirin-treated PGH_2 synthase II (0.8 μM) was reacted with 200 μM ethyl hydrogen peroxide, and the formation of compound I and compound II was followed on the stopped-flow spectrometer. (A) Absorbance spectra of the Soret band region for aspirin-treated PGH_2 synthase II and (B) kinetic trace of changes in absorbance at 411 nm. Aspirin-treated PGH_2 synthase II (0.8 μM) was reacted with 50 μM arachidonic acid, and the formation of a unique spectral intermediate was followed on the stopped-flow spectrometer. (C) Absorbance spectra of the Soret band region for PGH_2 synthase II and (D) kinetic trace of changes in absorbance at 446 nm. In parallel experiments, arachidonic acid-dependent oxygen uptake was measured. Oxygen consumption was monitored by computerized Clark-styled oxygen electrodes. (E) Change in the amount of oxygen in the chamber over time (○−○) and the first derivative of the change in amount of oxygen in the chamber (□−□). (F) the first derivative of the change in amount of oxygen in the chamber in the first 4 s (□−□).

treated PGH_2 synthase II would form spectral intermediates during the 15-dioxygenation of arachidonic acid, this enzyme form was incubated with arachidonic acid and spectral changes were monitored by stopped-flow spectrometry. In parallel experiments, arachidonic acid-dependent oxygen uptake was monitored using oxygen electrodes. In these experiments, the formation of a unique spectral intermediate with an absorbance maxima at 446 nm was observed by stopped-flow spectrometry within 3 ms (Figure 5, panels C and D). This species is present at the time when oxygen consumption is catalyzed by the aspirin-treated PGH_2 synthase II, which was found to occur within 200 ms after the addition of arachidonic acid (Figure 5, panels E and F). Thus, the unique spectral intermediate is kinetically competent to participate in the reaction. The time course for the 15-dioxygenation of arachidonic acid is essentially identical to

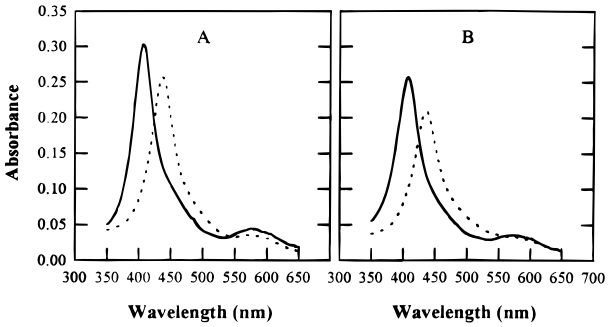


FIGURE 6: Ferric and ferrous holo-PGH_2 synthase I and II. The absorbance spectra through the Soret band region was measured for PGH_2 synthase I and II and for chemically prepared ferrous-PGH_2 synthase I and II by difference spectroscopy using a Hewlett-Packard 8452 diode array spectrometer. (A) To generate holo-PGH_2 synthase I, apo-PGH_2 synthase I (2.0 μM) was incubated with excess hematin (20.0 μM). Using the absorbance spectra of 18.0 μM hematin as the blank, the absorbance spectra of the soret region of PGH_2 synthase was measured (—). To generate the ferrous form, holo-PGH_2 synthase I was reacted with 1.1 mM Na_2SO_4 in 100 mM Tris-HCl, 15 μM diethyldithiocarbamic acid under argon in an anaerobic cuvette at 25 °C, and its spectrum was measured against a blank containing 18.0 μM hematin that had also been reacted with 1.1 mM Na_2SO_4 (···). (B) The spectrum of holo-PGH_2 synthase II (2.0 μM) (—) and of the ferrous form (···) were determined in an identical manner.

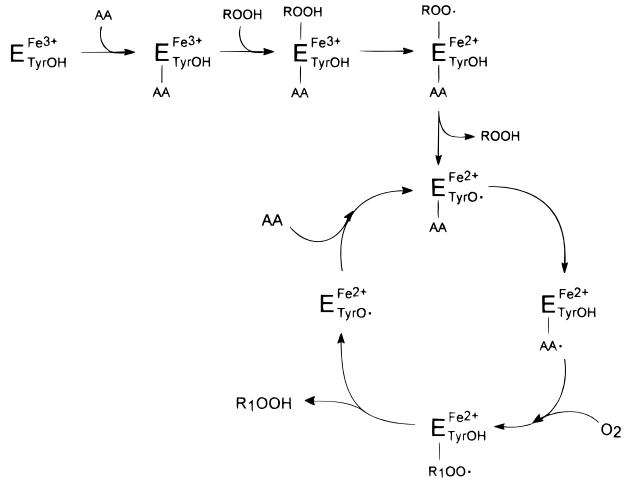
the time course of arachidonic acid dioxygenation catalyzed by untreated PGH_2 synthase II (panels E and F of Figures 2 and 5).

Ferrous (Fe^{2+}) protoporphyrin-IX has a major absorbance peak at 440 nm (Lambeir et al., 1985). We have generated the ferrous form by incubating holo-PGH_2 synthase I and holo-PGH_2 synthase II with sodium dithionite and found that its λ_{max} is 445 nm (Figure 6). The spectral properties of the Fe^{2+} form are strikingly similar to the new spectral intermediate observed when aspirin-treated PGH_2 synthase II was incubated with arachidonic acid. This would suggest that this intermediate may represent a Fe^{2+} form of PGH_2 synthase II. Since this species has been shown to be kinetically competent, the 15-dioxygenation of arachidonic acid catalyzed by aspirin-treated PGH_2 synthase II must be occurring via this Fe^{2+} intermediate. The implications of the involvement of a Fe^{2+} intermediate in a single dioxygenation event are now discussed.

CONCLUSIONS

We have proposed a mechanism by which the Fe^{2+} intermediate is involved in the single dioxygenation event (Scheme 3). We have demonstrated that aspirin-treated PGH_2 synthase II exhibits a normal peroxidase activity and compound I and compound II are detected during the reaction. We have also demonstrated that the Fe^{2+} intermediate is detected when the aspirin-treated enzyme is reacted with arachidonic acid. These observations provide the first evidence that the peroxidase reaction and the events initiating the dioxygenase event function independently. These observations would require that the initial binding of arachidonic acid to the aspirin-treated enzyme determines that the ferrous intermediate will form. If a peroxidase substrate binds, compound I and compound II will form. Following binding of arachidonic acid, the next step would require the oxidation of a hydroperoxide to yield a peroxy radical at the expense of reducing Fe^{3+} in protoporphyrin-

Scheme 3: Proposed Mechanism for the 15-Dioxygenation of Arachidonic Acid Catalyzed by Aspirin-Treated PGH_2 Synthase II[a]



[a] Key: E = aspirin-treated PGH_2 synthase II; AA = arachidonic acid; Tyr = tyrosine 371 (equivalent to Tyr 385 in PGH_2 synthase I); ROOH = initiator hydroperoxide; R_1OOH = 15(*R*)-HPETE.

IX of aspirin-treated PGH_2 synthase II to Fe^{2+}−protoporphyrin-IX (ferrous intermediate). The peroxy radical would then generate the tyrosyl radical. This tyrosyl radical could then activate arachidonic acid to initiate oxygen insertion to yield the peroxy radical of 15(*R*)-HPETE. This radical would dissociate from the enzyme as the hydroperoxide regenerating a resident tyrosyl radical. In this manner, an autocatalytic cycle is established for the subsequent activation of fresh arachidonic acid. This mechanism takes into account the role of an initial arachidonic acid binding event, the role of initiating hydroperoxides, the role of the tyrosyl radical, the formation of the Fe^{2+} intermediate, and the autocatalytic nature of the reaction.

The necessity of compound I and compound II to initiate the cyclooxygenase reaction of PGH_2 synthase II is contradicted by the observation that the formation of compound I and compound II during reactions with arachidonic acid is observed sooner than they are observed during reactions with either ethyl hydrogen peroxide or PPHP. This would imply that the formation of compound I and compound II is not the result of an initiating reaction in which contaminating species of hydroperoxide present in arachidonic acid are cleaved. Further, this would imply that compound I and compound II do not precede the cyclooxygenase reaction.

The discovery of a ferrous intermediate during aspirin-treated PGH_2 synthase II catalysis has implications toward the overall enzymatic mechanism of both PGH_2 synthases. Since it is unlikely that 11- and 15-dioxygenation occur via more than one mechanism, these data question the involvement of compound I and compound II in the cyclooxygenase reaction of both synthases. Previous studies have indicated that a hydroperoxide is required to initiate the cyclooxygenase reaction, albeit at very low concentrations (Kulmacz & Wang, 1995), while our findings have demonstrated that the hydroperoxide does not undergo peroxidative cleavage to form compound I and compound II in an initiating reaction. We therefore suggest that a peroxy radical is formed from the hydroperoxide and this initiates the cyclooxygenase reaction in both synthases as originally proposed by Hemler and Lands (1980).

In conclusion, this work represents the first stopped-flow studies to be described for PGH_2 synthase II. In the pre-steady-state, the peroxidase activity is clearly slower than the cyclooxygenase activity in this enzyme, thereby permitting spectral intermediates associated with the latter activity to be detected easily. In PGH_2 synthase I, the peroxidase activity occurs faster than the cyclooxygenase activity which would dictate that the reaction chamber will always contain a mixture of the holoenzyme in different iron oxidation states with arachidonic acid (ferric, compound I, compound II, and, perhaps, ferrous), making detection of the ferrous intermediate difficult. This would lead to the conclusion that, if single activity forms of PGH_2 synthase I were available (e.g., cox-only), perhaps the ferrous intermediate would be observed. Toward that end, we have developed a method to chemically modify PGH_2 synthase I to generate a cyclooxygenase-only form of the enzyme [see Tang et al. (1995)]. When cyclooxygenase-only PGH_2 synthase I is used in stopped-flow experiments with arachidonic acid, the ferrous intermediate of the enzyme is again observed. This finding is currently the subject of a second manuscript.

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