Spectral Properties of the Higher Oxidation States of Prostaglandin H Synthase*

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Prostaglandin H (PGH) synthase reacts with organic hydroperoxides and fatty acid hydroperoxides on a millisecond time scale to generate an intermediate that is spectrally similar to compound I of horseradish peroxidase. Compound I of PGH synthase is converted to compound II within 170 ms. Compound II decays to resting enzyme in a few seconds. Thus, the peroxidase reaction of PGH synthase appears to involve a cycle of native enzyme, compound I, and compound II, typical of heme-containing peroxidases. The Soret absorption maxima of compound I1 appears to occur at 412 nm but a small amount of compound I1 may be present. Soret maxima occur at 420, 433, and 419 for compound II, the ferrous enzyme, and the oxyferrous enzyme (compound III), respectively. Rapid scan analysis of the reaction of PGH synthase with arachidonic acid reveals the absorbance of compound II but no evidence for ferrous or oxyferrous enzyme.

PGH1 synthase catalyzes the first two reactions in the biosynthesis of prostaglandins. The cyclooxygenase component oxygenates arachidonic acid to PGG2, a hydroperoxy endoperoxide, and the peroxidase component reduces the hydroperoxy group of PGG2 to an alcohol, PGH2 (Equation 1) (1). PGH synthase is a membrane-bound protein and has been purified to apparent electrophoretic homogeneity from bovine and ovine seminal vesicle microsomal preparations (2–4). The purified enzyme, a homodimer of 70,000-dalton subunits, contains both cyclooxygenase and peroxidase activities (2, 4). It is a hemoprotein and the heme prosthetic group is required for both activities (2, 5).

The peroxidase component of PGH synthase reduces several different hydroperoxides while oxidizing a variety of reducing substrates (1, 6). Numerous studies of peroxidative oxidation by PGH synthase are interpreted by assuming it is a conventional heme peroxidase but direct evidence is lacking. Specifically, the higher oxidation states of heme peroxidases (e.g., compounds I and II of horseradish peroxidase) have not been detected. O’Brien and Rahimtulla (7) reported a difference spectrum in the reaction of rat seminal vesicle microsomes with arachidonic acid and Nastainczyk et al. (8) reported a similar difference spectrum for the purified enzyme with a peak at 430 nm in the Soret region and peaks at 525 and 555 nm in the visible region. They suggested this species represented an intermediate analogous to compound I of horseradish peroxidase. Prolonged exposure to hydroperoxides bleaches the heme (7).

We have utilized rapid-scan spectrophotometry to record the absolute spectra of peroxidase intermediates generated in the millisecond time range. Species are detectable that are very similar to compounds I and II of horseradish peroxidase. Compound II is responsible for difference spectra previously attributed to compound I. Experiments with arachidonic acid and PGH synthase indicate that these intermediates are formed as a result of the cyclooxygenase reaction and that no other spectrally detectable species are generated.

MATERIALS AND METHODS
PGH synthase was purified from ram seminal vesicle microsomes as previously described (9). The enzyme preparation was greater than 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme was 44–50% holoenzyme based on cyclooxygenase activity in the presence and absence of exogenous hematin and on absorbance at 410 nm using a molar absorptivity of 123 nmol−1 cm−1 (10). The enzyme was used without reconstitution with hematin. It exhibited cyclooxygenase activity of 22 μmol of arachidonic acid oxidized per mg of protein/min in the absence of exogenous hematin and 49 μmol of arachidonic acid oxidized per mg of protein/min in the presence of 1 μM hematin. Enzyme concentrations were estimated by absorbance at 410 nm. Protein was estimated using the Bio-Rad assay with bovine serum albumin as the standard (Bio-Rad). Unless otherwise indicated, aliquots of the purified enzyme were desalted on Sephadex G-25 equilibrated in 100 mM phosphate (pH 7.7), 30% glycerol to remove diethylthioldicarbonate present for stabilization during the purification and storage of the enzyme.

15-Hydroperoxyeicosatetraenoic acid was prepared from arachidonic acid using soybean lipoygenase by the method of Funk et al. (11). PPHP was a generous gift from Paul Weller (Department of Chemistry, Wayne State University). Arachidonic acid was purchased from NuChek Prep. Substrate samples were dissolved in 100 mM phosphate (pH 7.7), 30% glycerol. Hydrogen peroxide (30%) was purchased from Fisher, m-chloroperoxybenzoic acid from Aldrich, and indomethacin from Sigma. Stock solutions of PPHP and indomethacin were prepared in methanol, and m-chloroperoxybenzoic acid in acetone; both were diluted (at least 100-fold) in glycerol/phosphate buffer.

Absorption measurements were performed with a Cary 219 spectrophotometer. Rapid scan spectra were recorded with a Union Giken RA601 Rapid Reaction Analyzer equipped with a 1-cm cell thermostated at 5 °C ± 0.5 °C. The absorption spectra were measured by means of a multichannel photodiode array and memorized in a digital computer system (Sord M200 Mark III). The analogue replica was plotted on an X-Y recorder. The dead time of the flow apparatus was 4 ms. Regions (96 nm) were scanned from 360 to 680 nm with 10 nm overlap in all regions.

In experiments with reduced PGH synthase, buffer and enzyme solutions were made anaerobic by flushing with argon (Zero Gas, Canadian Liquid Air) purified by passage through an oxygen trap (Alltech Associates). Enzyme solutions were reduced by adding micromolar amounts of a concentrated sodium dithionite solution (0.1 M in 1 mM NaOH) to a final concentration of 100 or 200 μM (12). The

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1 The abbreviations used are: PGH, prostaglandin H; PPHP, 5-phenylpent-4-enyl-1-hydroperoxide.
reduction was performed immediately before transferring the enzyme to the rapid scan apparatus.

RESULTS AND DISCUSSION

When 11 eq of PPHP are added to native PGH synthase, a good yield of compound I is observed (Fig. 1) \( (k = 1.3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) \) as judged by the comparable behavior of horseradish peroxidase with several reactive peroxides. Compound I of PGH synthase then decays spontaneously to a species with a compound II-like spectrum (Fig. 2), again in accord with the behavior of many other peroxidases \( (k = 1.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \).

The visible region spectrum is particularly relevant, where the \( \alpha \) and \( \beta \) bands are located close to those found for compound II of horseradish peroxidase and distinct from those of horseradish peroxidase compound III (oxyferrous peroxidase). Spectral data for PGH synthase with comparisons to horseradish peroxidase are summarized in Table I.

Spontaneous decay of compound II of PGH synthase back to the native enzyme is then observed with a small loss in absorbance indicating some heme destruction (data not shown) \( (k < 0.1 \text{ s}^{-1}) \). The rate of disappearance of the compound II spectrum increases with increasing [PPHP], which may indicate that PPHP can also act as a reducing substrate. The enzyme can be recycled many times by subsequent addition of equimolar amounts of PPHP. A few percent destruction of heme is observed upon completion of each cycle.

The addition of a 10-fold excess of 15-hydroperoxyeicosatetraenoic acid leads to a smaller yield of compound I of PGH synthase and on a slower time scale than the reaction of an equimolar amount of PPHP (data not shown) \( (k = 1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \). 15-Hydroperoxyeicosatetraenoic acid is therefore a poorer oxidizing substrate than PPHP. When either hydrogen peroxide or m-chloroperoxybenzoic acid is added to native PGH synthase, very little change is recorded on the millisecond- or longer time scale in the Soret spectral region. Thus, two excellent oxidizing substrates for horseradish peroxidase do not produce spectral intermediates with PGH synthase, indicating profound differences in the distal active site regions.

Upon addition of a 10-fold molar excess of arachidonic acid to an aerated solution of PGH synthase, no compound I formation is detected. Rather over the time interval of 2 to 26 s after mixing, the conversion of native enzyme to compound II is observed (Fig. 3) \( (k = 10^6 \text{ M}^{-1} \text{s}^{-1}) \). Spontaneous decay of compound II back to the native enzyme is accompanied by 20% loss of the initial absorbance. Preincubation of the enzyme with a 20-fold molar excess of indomethacin, a selective cyclooxygenase inhibitor (13), inhibited both the formation of compound II and the destruction of heme by arachidonic acid. Under similar conditions, indomethacin had no effect upon the formation of compounds I and II from the enzyme by PPHP.

Our spectrum of the ferrous enzyme differs from that published previously which was obtained with reconstituted en-

\[ \text{FIG. 1. Reaction between PGH synthase and PPHP: formation of compound I.} \]

Concentrations after mixing were 1.5 \( \mu \)M PGH synthase for the Soret spectra, 12.1 \( \mu \)M PGH synthase for the visible spectra, and 17 \( \mu \)M PPHP for each region. Spectra in the Soret region are shown for native enzyme, 1 and 3 ms after mixing. Spectra in the visible region are shown for native enzyme, 2 and 16 ms after mixing. The arrows indicate the direction of the absorbance change with increasing time. The broken line is the native enzyme spectrum.

\[ \text{FIG. 2. Reaction between PGH synthase and PPHP: formation of compound II.} \]

Spectra in the Soret region are shown at 11, 22, and 132 ms after mixing. Spectra in the visible region are shown at 16, 34, and 166 ms after mixing. The arrows indicate the direction of the absorbance change with increasing time. The broken line is the native enzyme spectrum.

\[ \begin{align*}
\text{TABLE I} \\
\text{Comparison of spectral properties of PGH synthase and horseradish peroxidase} \\
\hline
& \text{PGH synthase } \lambda_{max} & \text{Horseradish peroxidase } \lambda_{max}^* \\
\text{Soret} & \text{Visible} & \text{Soret} & \text{Visible} \\
\hline
\text{Ferric} & 412, 501, ~545sh, ~580sh, 654^* & 403 & 438, 640 \\
\text{Compound I} & 412, Featureless & 400 & ~529sh, ~577, 622, 661 \\
\text{Compound II} & 420, 527, 557 & 420 & 527, 554 \\
\text{Ferrous} & 433, 559, ~590sh & 440 & ~519sh, 555, ~580sh \\
\text{Oxyferrous} & 419, 545, 579 & 416 & 546, 583, 673 \\
\end{align*} \]

* From Ref. 18.

sh, shoulder.

zyme (14). In the visible region we observe a single peak at 559 nm with a shoulder at 590 nm. Upon mixing the ferrous enzyme with aerated buffer, a compound III (oxyferrous PGH synthase) spectrum is obtained (Fig. 4 and Table I). The lack of an isosbestic point and subsequent loss of 15% absorbance indicates that heme destruction is occurring in this time

\[ k_{1-n} \]
between the oxyferrous PGH synthase that, in terms of its reaction cycle, PGH synthase is a typical
intermediate. The spectra were collected during 5 ms in 96-nm wavelength intervals. The spectra shown were taken 1.2, 7.8, and 16.6 s after mixing. The arrows indicate the direction of the absorbance change with increasing time. Spectra were collected during 100 ms in 96-nm wavelength intervals.

![Absorbance vs Wavelength](chart.png)

**Fig. 3. Spectral intermediates during the reaction of PGH synthase with arachidonic acid.** Concentrations after mixing were 1.1 μM PGH synthase in the Soret region, 3.2 μM PGH synthase in the visible region, and 17 μM arachidonic acid for each region. The spectra shown were taken 1.2, 7.8, and 16.6 s after mixing. The arrows indicate the direction of the absorbance change with increasing time. Spectra were recorded during 100 ms in 96-nm wavelength intervals.

interval. Independent measurements indicate that excess dithionite is being consumed in a reaction with molecular oxygen during this period. Heme destruction with excess dithionite and molecular oxygen has been utilized through several cycles to prepare the apoenzyme (15). After 800 ms, heme destruction ends and an isosbestic point is observed between the oxyferrous PGH synthase (I₁/₂ = 4.5 s) and its decay product, the native enzyme (Fig. 5). When 1.8 μM ferrous enzyme is mixed with air-saturated buffer containing 17 μM arachidonic acid, no change in the rate of compound III formation or decay is observed.

The studies summarized in Figs. 1 and 2 represent the first complete characterization of the peroxidase reaction of PGH synthase. Intermediates are detected that are spectrally analogous to those of other heme peroxidases, which indicates that, in terms of its reaction cycle, PGH synthase is a typical heme peroxidase. The similarities to horseradish peroxidase are striking (Table I) although additional experiments are necessary to definitely establish the electronic properties of the higher oxidation states of PGH synthase. The decreased absorbance of compound I is indicative of a radical cation derivative of the heme. The identity of the heme of PGH synthase has been determined to be iron protoporphyrin IX by the pyridine hemochromogen method (16). The position of the absorbance maxima of compound II then may indicate that imidazole is the fifth ligand to the heme iron; however, differences in native enzyme and compound I spectra remain to be explained.

The slower rate of reaction of the enzyme with arachidonic acid relative to PPH is expected because of the reduced rate of hydroperoxide formation at 5°C. Under these conditions, the inability to detect compound I arises because the rate of its formation from arachidonic acid (reflecting the slow rate of PGG₂ formation) is slower than the rate of its consumption. An intriguing possibility is that arachidonate reacts with compound I, generating compound II and an arachidonate radical which reacts with O₂ to form PGG₂. What is very clear from these experiments is that ferrous and oxyferrous forms of the enzyme are not detectable during either the peroxidase or cyclooxygenase catalytic cycles. Thus, experimental evidence does not support the hypothesis that the enzyme cycles between Fe³⁺ and Fe²⁺ during cyclooxygenase catalysis.

**Fig. 4. Formation of oxyferrous (compound III) PGH synthase.** Ferrous PGH synthase was mixed with air-saturated buffer (100 mM phosphate, pH 7.7). Concentrations after mixing were 50 μM Na₂S₂O₄ and 190 μM O₂. The enzyme concentration was 1.4 μM for the Soret and 12.4 μM for the visible region. The enzyme solution contained diethyldithiocarbamate as a stabilizing agent. Spectra are shown for the ferrous enzyme (a), 60 ms (b), and 830 ms (c) after mixing. The spectra were collected during 5 ms in 96-nm wavelength intervals.

**Fig. 5. Decay of oxyferrous (compound III) PGH synthase.** Experimental conditions as in Fig. 4. The spectra shown are 1.5 and 6 s after mixing and the final (ferric) spectrum upon completion of the decay process. The arrows indicate the direction of the absorbance change with increasing time.

![Absorbance vs Wavelength](chart2.png)

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