Reactions of Prostaglandin Endoperoxide Synthase and Its Compound I with Hydroperoxides*

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The reactions of native prostaglandin endoperoxide synthase with structurally different hydroperoxides have been investigated by using kinetic spectrophotometric scan and conventional and sequential mixing stopped-flow experiments. The second order rate constants for compound I formation are \((5.9 \pm 0.1) \times 10^8 \text{M}^{-1} \text{s}^{-1}\) using \(t\)-butyl hydroperoxide as the oxidant, \((2.5 \pm 0.1) \times 10^9 \text{M}^{-1} \text{s}^{-1}\) for ethyl hydroperoxide and \((5.1 \pm 0.6) \times 10^9 \text{M}^{-1} \text{s}^{-1}\) for m-chloroperoxybenzoic acid at pH 7.0, 6.7 ± 0.2°C, and ionic strength 0.1 M. Sequential mixing, transient state experiments show for the first time that all hydroperoxides reduce compound I in a bimolecular reaction. Ethyl hydroperoxide, \(t\)-butyl hydroperoxide, and m-chloroperoxybenzoic acid react directly with compound I. The natural substrate prostaglandin G2 forms a transient complex with compound I before the reduction step occurs. Therefore, compound I initially transforms to compound II, not to the compound I-tyrosyl radical. Second order rate constants for the reactions of compound I are \((2.9 \pm 0.2) \times 10^5\) for \(t\)-butyl hydroperoxide, \((3.5 \pm 0.5) \times 10^5\) for hydrogen peroxide, \((4.2 \pm 0.2) \times 10^6\) for ethyl hydroperoxide, and \((4.2 \pm 0.3) \times 10^6\) for m-chloroperoxybenzoic acid, all in units of \(\text{M}^{-1} \text{s}^{-1}\) and same conditions as for compound I formation. The rate of reaction of prostaglandin G2 with compound I, calculated from the ratio of \(k_{\text{cat}}\) to \(K_m\) obtained from the saturation curve, is \((1.0 \pm 0.2) \times 10^6 \text{M}^{-1} \text{s}^{-1}\) at 3.0 ± 0.2°C. Results are discussed in the context of the current state of knowledge of the mechanisms of the cyclooxygenase and peroxidase reactions of prostaglandin endoperoxide synthase.

Prostaglandin endoperoxide synthase (PGH synthase) catalyzes the first committed step of the biosynthesis of prostaglandins and thromboxanes from arachidonic acid (for recent reviews, see Refs. 1–3). Two distinct isoforms of PGH synthase are found that are differently expressed in specific tissues (4–8). The cDNA isolation and the elucidation of mechanisms by which the PGH synthase gene transcription is regulated are the basic objectives of the most recent molecular biological studies (9–14). Substantial information has become available from recent x-ray crystallographic (15), site-directed mutagenesis–EPR (16, 17), and kinetic (18, 19) studies. Despite these efforts, progress has been limited in clarifying the role of the higher oxidation states of the enzyme and the number of tyrosyl radicals implicated in the catalytic mechanism.

Upon reaction with organic hydroperoxides, two distinct spectral intermediates of PGH synthase are formed resembling compound I and compound II peroxidase intermediates (20). The heme Fe(III) of the native enzyme is oxidized by the peroxygen and an Fe(V)=O porphyrin π cation radical (compound I) is formed. Compound I is readily converted to a second intermediate, which we shall call intermediate II. The oxidation state of intermediate II could be assigned either to an Fe(V)=O species with no enzymatic free radical (compound II) or to a Fe(V)=O-tyrosyl radical species (compound I-tyrosyl radical) as in compound I of cytochrome c peroxidase (22). Both Fe(V)=O species could be formed under certain conditions, but how they are related to each other is a crucial and as yet unresolved problem in the PGH synthase catalytic mechanism.

The detection of tyrosyl radicals has been considered to be key evidence for a postulated reaction mechanism in which the compound I-tyrosyl radical species abstracts a hydrogen atom from arachidonic acid, which initiates the oxygenation reaction and generates the tyrosyl residue (23). If the oxygenated arachidonic acid radical, the PGG2, abstracts a hydrogen atom from the tyrosyl residue to become PGG2, then the tyrosyl radical is regenerated and a chain reaction could occur. For every compound I converted to the Fe(V)=O-tyrosyl radical another chain could be started, so this mechanism is properly called a branched-chain mechanism. The branched-chain mechanism could lead to the accumulation of large amounts of PGG2, not PGH2, which is the precursor of all other prostaglandins.

It has been shown that the branched-chain mechanism cannot explain coupled cyclooxygenase and peroxidase activities when an excess of a substrate electron donor is present; yet, the chain reaction cannot be excluded under conditions when the peroxidase reaction is rate-limiting, i.e. at low concentration or in the absence of a peroxidase electron donor (18). Under such conditions, however, the inactivation of the enzyme is severe, and the tyrosyl radical(s) could be participating in these processes.

An alternative non-chain mechanism involves tyrosyl radical formation at a later stage in the reaction (18, 19). The present study was undertaken to test the occurrence of the critical elementary reaction in the tyrosyl radical chain reaction model (23), the spontaneous formation of the compound I-tyrosyl radical from compound I. Evidence will be shown that when a hydroperoxide is present, the compound I-tyrosyl radical is not formed from compound I. Compound I rather abstracts a hydrogen atom from the hydroperoxide to generate compound II and hydroperoxyl radical, as can occur with myeloperoxidase (24). The compound I-tyrosyl radical could then be formed from...
compound II and hydroperoxyl radical as previously suggested (18, 19).

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following sources: arachidonic acid, Nu-chek-Prep; phenol, British Drug House; DE-53 ion exchange chromatography gel, Whatman; Tween 20, J. T. Baker Chemical Co.; m-CI PBA (85%), Aldrich; ETOOH (10–12%, aq), Polysciences, Inc.; t-BuOOH (70%, aq), Sigma; H₂O₂ (30%, aq), Fisher; PGG₂ (>95%), Cayman Chemical; superoxide dismutase from bovine erythrocytes, Sigma; bovine serum albumin, Sigma; and horseradish peroxidase (grade I), Boehringer Mannheim.

The concentration of horseradish peroxidase was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02 × 10⁵ M⁻¹ cm⁻¹ (25). The concentration of hydroperoxidase stock solutions was determined by the horseradish peroxidase assay (26).

PGH synthase, isolated from sheep seminal vesicles (27, 28), consisted of approximately a 50/50 mixture of apo- and holoenzyme and had a specific activity of 40 μmol of arachidonic acid (mg of protein/min)⁻¹ in the presence of 1 μM hematin and 1 mM phenol. Protein content was determined by the Bio-Rad protein assay. The concentration of horseradish peroxidase stock solutions was reconstituted by adding an amount of hematin equal to the amount of apoenzyme. The final heme concentration of the enzyme, determined after keeping the enzyme sample on ice for 10–15 min, was usually 23–25 μM.

Hydroperoxide Purity Test—The presence of impurities that may serve as exogenous peroxidase electron donors in all hydroperoxide solutions (ROOH) was tested using horseradish peroxidase. A 1:1 mixture of horseradish peroxidase was mixed with an equal amount of hydroperoxide in 0.1 M phosphate buffer, pH 7.03, at room temperature. The horseradish peroxidase was mixed with an equal amount of hydroperoxide (ROOH) and the rate of formation of intermediate II was monitored by following the rate of formation of intermediate II at 426 nm, typical time course trace and exponential curve fit obtained at 414 nm. Mixture contained 0.5 μM enzyme and 55.4 μM ETOOH; kₗobs = 177 ± 3 s⁻¹, and total ΔA = 0.011.

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Kinetic Experiments—The kinetic experiments were performed on an Applied Photophysics SX.17 MV (Micro-Volume)Stopped-flow Reaction Analyzer. If not specified otherwise, the experiments were conducted at 6.7 ± 0.2 °C in phosphate buffer, pH 7.03, at an ionic strength of 0.1 M, with buffer contributing 0.01 M and K₂SO₄ contributing 0.09 M. The pH jump technique was applied to avoid denaturation of the enzyme before the reaction with hydroperoxide (30). The enzyme solutions were buffered but had an ionic strength of 0.1 M maintained by K₂SO₄. For a pH dependence study, 0.1 M citrate (pH 3.56–5.95), phosphate (pH 7.03–8.00), and carbonate buffers (pH 8.00–9.06) were used. The pH dependence study was performed only for the reaction with H₂O₂ at 4.0 ± 0.2 °C.

Standard Mode Stopped-flow Measurements (Pre-steady State)—In standard mode experiments two solutions are directly mixed and passed to the observation cell. One drive syringe was filled with enzyme solution. The other syringe was filled with hydroperoxide. At least a 10-fold excess of hydroperoxide was used to maintain pseudo-first order conditions. Compound I formation was monitored by the disappearance of native enzyme at 414 nm, an isosbestic point between compound I and compound II. The subsequent compound II disappearance was monitored by following the rate of formation of intermediate II at 426 nm, an isosbestic point between native enzyme and compound I (31–33). The intermediate II (compound II or compound I-tyrosyl radical) disappearance was also monitored at 426 nm.

Sequential Mode Stopped-flow Measurements (Transient State)—When shifting to sequential mixing, a reconfiguration is required of both the hardware and software of the SX.17 MV instrument. Four syringes, A, B, C, and F, are used. Syringes A and B are pushed by drive 1, and after an appropriate delay time, syringes C and F are pushed by drive 2. As recommended by the manufacturer, the positions of the pre-mixing ram (drive 1) and the flush ram (drive 2) were adjusted to provide an ideal total volume of 220 and 180 μl, respectively, to provide accurate mixing conditions. Syringe A was filled with 4x the final concentration of enzyme; syringe B with 4x the final concentration of m-CI PBA; syringe C with 2x the final concentration of an appropriate hydroperoxide, one of H₂O₂, ETOOH, t-BuOOH, m-CI PBA, or PGG₂; and syringe F (flush) with the buffer. Drive 2 was adjusted to be activated 60–70 ms after drive 1. This results in an aging time of 55–65 ms (total time that enzyme and m-CI PBA are allowed to react in the

RESULTS

Rate Constants for Compound I Formation—The reaction of native enzyme (E) with a hydroperoxide (ROOH) and the rate of compound I formation (d[E-I]/dt) are defined in Reaction 1 and Equations 1 and 2:

\[ \text{E + ROOH} \rightarrow \text{E-I + ROH} \]

\[ \text{REACTION 1} \]

\[ \text{d[E-I]/dt} = k₁ \text{[E][ROOH]} \quad \text{(Eq. 1)} \]

\[ k_{\text{lobs}} = k₁ \text{[ROOH]} \quad \text{(Eq. 2)} \]

The kinetic traces at 414 nm were single-exponential, and the pseudo-first order rate constants kₗobs were linear functions of [ROOH] (the plot and a kinetic trace for ETOOH are shown in Fig. 1). The bimolecular rate constants k₁ were calculated from the slopes of the straight-line plots of kₗobs versus [ROOH].

\[ \text{FIG. 1. Plot of observed pseudo first order rate constant for the formation of PGH synthase compound I (}k_{\text{lobs}}\text{) versus the concentration of ethyl hydroperoxide.} 0.5 \mu\text{M solution of PGH synthase in 0.1 M phosphate buffer, pH 7.03, was mixed with different [ETOOH] at 6.7 ± 0.2 °C. Insets, typical time course trace and exponential curve fit obtained at 414 nm. Mixture contained 0.5 μM enzyme and 55.4 μM ETOOH; } k_{\text{lobs}} = 177 ± 3 \text{ s}^{-1}, \text{ and total ΔA = 0.011.} \]
Table I

| Peroxide                        | $k_2$ (s$^{-1}$) |
|--------------------------------|------------------|
| Hydrogen peroxide$^a$           | $(4.4 \pm 0.2) \times 10^6$ |
| Ethyl hydroperoxide             | $(2.5 \pm 0.1) \times 10^6$ |
| t-Butyl hydroperoxide           | $(5.9 \pm 0.1) \times 10^4$ |
| m-Chloroperoxycarboxylic acid   | $(5.1 \pm 0.6) \times 10^7$ |
| Prostaglandin G$_2$-Cl$^a$      | $(1.4 \pm 0.2) \times 10^7$ |

$^a4.2 \pm 0.2$ °C (M. Bakovic and H. B. Duford, submitted for publication).

$^b1.0 \pm 0.5$ °C (Ref. 23).

Results indicating values for different hydroperoxides are presented in Table I, together with a published value for PGG$_2$ (23). The results indicate that the aliphatic hydroperoxides H$_2$O$_2$, EtOOH, and t-BuOOH are significantly poorer substrates for native enzyme than the aromatic hydroperoxide m-Cl PBA. The rate constants for compound I formation decrease in the order m-Cl PBA $\gg$ EtOOH $>$ H$_2$O$_2$ $>$ t-BuOOH. For m-Cl PBA, $k_1$ was two to three orders of magnitude higher ($5 \times 10^7$ M$^{-1}$ s$^{-1}$) than for other hydroperoxides and similar to the value obtained with the natural substrate PGG$_2$ (Table I).

The Effect of Different Hydroperoxides on Intermediates II Formation Under Pre-steady State Conditions—The rate of formation of intermediate II from compound I ($k_{2,obs}$) was followed by an increase in absorbance at 426 nm as shown in the inset to Fig. 2. The dependence of the pseudo-first order rate constant for intermediate II formation, $k_{2,obs}$ on [ROOH] is shown in Figs. 2 and 3. The faster reacting substrates, EtOOH and m-Cl PBA, had a linear dependence of $k_{2,obs}$ on [ROOH] (Fig. 2). For the poorer substrates, H$_2$O$_2$ and t-BuOOH, saturation curves were obtained (Fig. 3). Rate data are summarized in Table II.

The results show that the faster phase in the PGH synthase intermediate II formation is not a function of pH. Similar pH independence was obtained by plotting log $k_{cat}/K_m$ against pH (data not shown).

Superoxide dismutase slightly increased the first, fast phase in intermediate II formation but did not eliminate the second, slow phase even at 10 μM concentration. Bovine serum albumin showed a similar effect to that of superoxide dismutase (data not shown). Because of poor reproducibility, the functional relationship between the rate of the slower phase and [H$_2$O$_2$] could not be ascertained. At some pH values, this relationship was linear, but at many other pH values, the relationship could not be established.

Reactions with Horseradish Peroxidase as a Purity Test for Hydroperoxides—The quality of the hydroperoxide preparations was tested by reaction with horseradish peroxidase, which provides a sensitive test for the presence of electron-donating impurities. The reactions of other hydroperoxides with horseradish peroxidase were compared with that of H$_2$O$_2$, EtOOH, m-Cl PBA, as well as H$_2$O$_2$ formed horseradish peroxidase compound I quantitatively by using stoichiometric amounts of the enzyme and hydroperoxide. No compound II from the newly formed horseradish peroxidase compound I prepared with EtOOH, m-Cl PBA, or H$_2$O$_2$ appeared in 5 min at room temperature, indicating that no electron donors were present in the hydroperoxide preparations. More than a 20-fold excess of t-BuOOH was necessary to form completely horseradish peroxidase compound I. However, no conversion of horseradish peroxidase compound I to horseradish peroxidase compound II was observed even at very high [t-BuOOH], indicating that no reductants of compound I were present.
tional test for solid m-Cl PBA, the rate constant $k_{2,obs}$ was determined for both crude and purified preparations. No difference in kinetic performance was obtained (Fig. 2), again indicating that no secondary reactions occurred. Nonetheless, the purified m-Cl PBA was used in the remainder of the experiments.

**Formation of Compound I from Stoichiometric Amounts of PGH Synthase and m-Cl PBA**—Here, we describe the most suitable way of obtaining a high yield of compound I. Slightly more than a stoichiometric amount of m-Cl PBA was enough to produce compound I of PGH synthase in high yield (Fig. 5A). At 6.7 °C and pH 7.03 (0.1 μM enzyme), the formation of compound I was complete within 50 ms. An isosbestic point occurs between the native enzyme and compound I at 422 nm. Compound I was stable for 50–80 ms and then was transformed at a slower rate to the second intermediate (Fig. 5B). This was accompanied by the spectral shift and increase in absorbance at longer wavelengths. The formation of the second intermediate was complete after 200 ms. The second intermediate had a maximum at 419 nm and an isosbestic point with compound I at 413 nm, i.e., similar to 414 nm reported initially (31-33).

The kinetic trace at 414 nm obtained with m-Cl PBA for the above conditions had two distinct transient phases separated by a steady state phase (Fig. 6). The fast decrease in absorbance is the formation of compound I, and slow increase in absorbance at longer times indicates the conversion of compound I to intermediate II. There was a short steady state period of 30 ms when compound I was stable, and the time between the native enzyme and compound I at 422 nm. There was a short steady state phase (Fig. 6). The fast decrease in absorbance is the formation of compound I, and slow increase in absorbance at longer times indicates the conversion of compound I to intermediate II. There was a short steady state period of 30 ms when compound I was stable, and the time.

### Table II

| Peroxide                  | $k_{2,obs}$ | Pre-steady state method | Transient state method |
|---------------------------|-------------|-------------------------|------------------------|
| Hydrogen peroxide         | $3.5 \pm 0.5 \times 10^6$ | Not reproducible        |                        |
| Ethyl hydroperoxide       | $(4.8 \pm 0.1) \times 10^6$ | $(4.2 \pm 0.2) \times 10^6$ |                        |
| t-Butyl hydroperoxide     | $(3.6 \pm 0.4) \times 10^6$ | $(2.9 \pm 0.2) \times 10^6$ |                        |
| m-Chloroperoxobenzoinic   | $(4.0 \pm 0.3) \times 10^6$ | $(4.2 \pm 0.3) \times 10^6$ |                        |
| Prostaglandin G2          | No reaction claimed$^a$ | $(1.0 \pm 0.2) \times 10^6$ |                        |
| Phenol                    | $8.3 \pm 0.2 \times 10^7$ |                          |                        |

$^a$ Calculated from the saturation curves shown in Fig. 3.
$^b$ Ref. 23.
$^c$ 3.0 ± 0.2 °C.

**Fig. 4.** The pH dependence for the faster phase in the formation of PGH synthase intermediate II using H$_2$O$_2$ as substrate. The experiments were performed under pre-steady state conditions. The faster rates, $k_{2,obs}$, measured at several pH, were plotted as a function of [H$_2$O$_2$] as shown in Fig. 3; the values of $k_{cat}$ and $K_m$ were obtained from the curve fits to the rectangular hyperbolic A = $k_{cat}$ or $K_m$.

**Fig. 5.** Spectral changes observed during the reaction of stoichiometric amounts of PGH synthase and m-Cl PBA. Kinetic scans were recorded in the 350–450-nm region (380–450-nm region is shown) at 6.7 ± 0.2 °C. The final concentrations were 0.50 μM enzyme and 6.5 μM m-Cl PBA. Broad arrows indicate the direction of the absorbance change with increasing time. A, spectra 0–80 ms after mixing, indicating that only compound I was formed; B, spectra 0–80 and 80–200 ms after mixing showing formation of the second intermediate. Isosbestic points between native enzyme and compound I (422 nm) and between compound I and the second intermediate (413 nm) are indicated.

**Fig. 6.** Kinetic trace at 414 nm showing the time delay between the formation and spontaneous decay of PGH synthase compound I. PGH synthase compound I was formed from the stoichiometric amounts of enzyme and m-Cl PBA as described in Fig. 5. The arrow indicates the time (60 ms) selected as a pre-set delay time in the sequential mixing experiments.

within that range, designated by an arrow in Fig. 6 (60 ms after the start of the reaction), was selected as an optimal time for studying the reactions of compound I by using the sequential mixing technique.

**Transient State Experiments on the Reaction of PGH Synthase Compound I with EtOOH, t-BuOOH, and m-Cl PBA**—Utilizing the sequential mixing method, the rate of intermedi-
change in absorbance at 426 nm obtained after mixing 0.5 mM EtOOH also present. For 

saturated state results with H₂O₂ were presented separately. The transient state results with H₂O₂ were exhibited different behavior, and these results will be pre-

amined reacted with compound I. The natural substrate PGG₂

thestandard stopped-flow experiments. All hydroperoxides ex-

sate II formation from compound I was studied directly as a function of [ROOH]. The reaction was followed at 426 nm as in the standard stopped-flow experiments. All hydroperoxides examined reacted with compound I. The natural substrate PGG₂ exhibited different behavior, and these results will be presented separately. The transient state results with H₂O₂ were not reproducible, apparently because of inactivation reactions, and they are not presented here.

Fig. 7 shows the linear plots of the observed pseudo-first order rate constant k₂,obs versus [ROOH] for the reactions of compound I with EtOOH, t-BuOOH, and m-Cl PBA under transient state conditions. A noticeable difference between the transient state and pre-steady state results was observed only with t-BuOOH. The plot of k₂,obs versus [t-BuOOH] from the transient state results is linear (Fig. 7), not curved as was observed in the pre-steady state (Fig. 3).

The linear plots had a common intercept at 15 s⁻¹ (Fig. 7). This value represents the rate for the spontaneous decay of compound I. The spontaneous rate was also determined in separate experiments in a multi-mixing assay in which a second peroxide was not added (curves a, inset to Fig. 7). Thus, only the 1:1.3 ratio of enzyme and m-Cl PBA were reacted. The results were in good agreement with the intercept value obtained in Fig. 7. The term “spontaneous” can be explained by the reduction of PGH synthase compound I by the stabilizing agent DDC to compound II (31–33). Small amounts of DDC are required for enzyme stability.

The linear kinetics and finite intercept for the reaction of PGH synthase compound I with EtOOH, t-BuOOH, and m-Cl PBA in the presence of DDC was obtained in the sequential mixing experiments (23). The increase in absorbance at 426 nm was a saturation function of [PGG₂]. An example is shown in Fig. 8. The results imply the formation of an inter-

mediate complex, E-I-PGG₂, between compound I and PGG₂–Transient state experiments with PGG₂ were performed at 3 °C for comparison with results of the previous published single-mixing (pre-

steady state) experiments (23). The increase in absorbance at 426 nm was a saturation function of [PGG₂]. An example is shown in Fig. 8. The results imply the formation of an inter-

mediate complex, E-I-PGG₂, between compound I and PGG₂ before the actual hydrogen atom transfer occurs, as shown in Reaction 4 and Equation 6:

\[
E-I + PGG_2 \rightarrow E-II + PGG_2^*.
\]
values decreased in the order PGG2, compound I and PGG2, The apparent second order rate constant for the reaction of the PGH synthase intermediate II at low and high hydroperoxides. The parameters were determined (Fig. 8) from the compound I-tyrosyl radical (total oxidation state of +5 but with iron in a +4 state). The elementary reactions of the native enzyme with hydroperoxides and compound II with peroxidase electron donors are well characterized (23, 31–33), but there is almost no data available for the reactions of compound I, which is the key intermediate in the catalytic mechanism. The extensive EPR studies on both the wild type and mutated enzymes have shown the existence of several tyrosyl radical species, the one formed from Tyr385 being assigned a role in catalysis and others in inactivation processes (16, 17, 34–36). The mechanism for the formation of the catalytically competent tyrosyl radical has been generally accepted as an intramolecular electron transfer process, mainly based on the single kinetic and modeling study with PGG2 (23).

Hydroperoxides are initiators of tyrosyl radical formation, and the oxygenation of arachidonic acid for which PGH synthase compound I formation is a prerequisite step (1–3). On the other hand, hydroperoxides inactivate the enzyme in the absence of peroxidase electron donors (37, 38). Our work is an extensive pre-steady state and transient state kinetic study with a series of structurally different hydroperoxides and PGG2, in which the primary goal was to deduce the role of hydroperoxides and compound I in tyrosyl radical formation.

Compound I Formation Rates and Stability—The magnitudes of the rate constants for compound I formation (Table I) suggest that native enzyme has preference for larger and more hydrophobic aromatic hydroperoxides. m-Cl PBA is an excellent substrate for the native enzyme with a rate constant for compound I formation similar to that for PGG2. This was surprising because from previous work it appeared that m-Cl PBA was a poor substrate for the enzyme (20), for which we have no explanation. Aliphatic hydroperoxides are poorer oxidants than m-Cl PBA, with EtOOH being a better substrate than H2O2 and t-BuOOH. t-BuOOH was the poorest substrate for the native enzyme, indicating that specific steric effects play a dominant role in this reaction.

We found that m-Cl PBA could be utilized for the quantitative formation of compound I and that compound I thus formed was sufficiently stable to study its reactions with other substrates using the sequential mixing technique (Figs. 5 and 6).

Reactions of Hydroperoxides with Compound I—The single mixing pre-steady state kinetics all indicated the surprising result that hydroperoxides were reacting with compound I (Figs. 2 and 3). We then proceeded with the more definitive transient state experiments using the sequential mixing technique.

The transient state results clearly establish that m-Cl PBA, EtOOH, and t-BuOOH exhibit bimolecular kinetics in their reactions with compound I and confirm results from the pre-steady state experiments that the initial product obtained is the key intermediate in the catalytic mechanism. The apparent reaction rate was determined (Fig. 8) from the compound I-tyrosyl radical (total oxidation state of +5 but with iron in a +4 state). The elementary reactions of the native enzyme with hydroperoxides and compound II with peroxidase electron donors are well characterized (23, 31–33), but there is almost no data available for the reactions of compound I, which is the key intermediate in the catalytic mechanism. The extensive EPR studies on both the wild type and mutated enzymes have shown the existence of several tyrosyl radical species, the one formed from Tyr385 being assigned a role in catalysis and others in inactivation processes (16, 17, 34–36). The mechanism for the formation of the catalytically competent tyrosyl radical has been generally accepted as an intramolecular electron transfer process, mainly based on the single kinetic and modeling study with PGG2 (23).

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For PGG2 \( \ll K_m \), the \( k_{2,\text{obs}} \) value approximates the pseudo-first order rate constant for the bimolecular reaction of compound I and PGG2 as in Equations 7 and 8:

\[
k_{2,\text{obs}} = \frac{k_{\text{cat}} \cdot [\text{PGG2}]}{K_m + [\text{PGG2}]} \quad \text{(Eq. 7)}
\]

\[
k_{2,\text{app}} = k_{\text{cat}} / K_m \quad \text{(Eq. 8)}
\]

The apparent second order rate constant for the reaction of compound I and PGG2, \( k_{2,\text{app}} \), obtained from Equation 8, is \( (1.0 \pm 0.2) \times 10^7 \text{M}^{-1} \text{s}^{-1} \) (Table I). As shown in Table I, the \( k_{2,\text{app}} \) values decreased in the order PGG2 > m-Cl PBA > EtOOH > H2O2 > t-BuOOH.

Enzyme Recycling and the Steady State Spectrum of Intermediate II—Fig. 9 shows that the second intermediate, spontaneously formed from compound I when the stoichiometric amounts of the native enzyme and m-Cl PBA reacted, returned to the native enzyme within 2 s. However, if an excess of EtOOH or m-Cl PBA were present, the steady state spectra of intermediate II (similar to spectrum a in Fig. 9A) were stable for more than 20 s, followed by a slow rate of decay that was independent of [ROOH]. The results for the decay of intermediate II in the presence of EtOOH are shown in Fig. 9B. Within experimental error, the observed pseudo-first order rate constant for the decay of intermediate II, \( k_{2,\text{obs}} \), was the same for EtOOH (0.08 ± 0.02 s\(^{-1}\)) and for m-Cl PBA (0.11 ± 0.03 s\(^{-1}\)) (data not shown). The decay rate of intermediate II was not measured for the other hydroperoxides.

**DISCUSSION**

Background—Studies of the PGH synthase reaction mechanism have been hindered by fast enzyme inactivation, the instability of enzyme higher oxidation states, and the existence of enzyme transients that share the same oxidation state of iron. Although compound I of PGH synthase exhibits the classic drop in absorbance from that of the native enzyme typical of all peroxidases, the subsequent reactions of compound I have been clouded because of inability to distinguish spectroscopically compound II (iron oxidation state of +4) from the compound I-tyrosyl radical (total oxidation state of +5 but with iron in a +4 state). The elementary reactions of the native enzyme with hydroperoxides and compound II with peroxidase electron donors are well characterized (23, 31–33), but there is almost no data available for the reactions of compound I, which is the key intermediate in the catalytic mechanism. The extensive EPR studies on both the wild type and mutated enzymes have shown the existence of several tyrosyl radical species, the one formed from Tyr385 being assigned a role in catalysis and others in inactivation processes (16, 17, 34–36). The mechanism for the formation of the catalytically competent tyrosyl radical has been generally accepted as an intramolecular electron transfer process, mainly based on the single kinetic and modeling study with PGG2 (23).

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For PGG2 \( \ll K_m \), the \( k_{2,\text{obs}} \) value approximates the pseudo-first order rate constant for the bimolecular reaction of compound I and PGG2 as in Equations 7 and 8:

\[
k_{2,\text{obs}} = \frac{k_{\text{cat}} \cdot [\text{PGG2}]}{K_m + [\text{PGG2}]} \quad \text{(Eq. 6)}
\]

The parameters \( k_{\text{cat}} \) (69 ± 6 s\(^{-1}\)) and \( K_m \) (70 ± 10 μM) were obtained from the plot of \( k_{2,\text{obs}} \) versus [PGG2] in Fig. 8. No corrections for the DDC spontaneous reaction \( (k_{\text{DDC}}) \) were made. At 3°C, \( k_{\text{DDC}} \) was 4 s\(^{-1}\). Since an excellent fit of the theoretical curve to the experimental data was obtained, it appears that the formation of the E-I-PGG2 complex (Reaction 4) prevented the reaction of compound I with the small amount of DDC.

For PGG2 \( \ll K_m \), the \( k_{2,\text{obs}} \) value approximates the pseudo-first order rate constant for the bimolecular reaction of compound I and PGG2 as in Equations 7 and 8:

\[
k_{2,\text{obs}} = \frac{k_{\text{cat}} \cdot [\text{PGG2}]}{K_m + [\text{PGG2}]} \quad \text{(Eq. 7)}
\]

\[
k_{2,\text{app}} = k_{\text{cat}} / K_m \quad \text{(Eq. 8)}
\]

The apparent second order rate constant for the reaction of compound I and PGG2, \( k_{2,\text{app}} \), obtained from Equation 8, is \( (1.0 \pm 0.2) \times 10^7 \text{M}^{-1} \text{s}^{-1} \) (Table I). As shown in Table I, the \( k_{2,\text{app}} \) values decreased in the order PGG2 > m-Cl PBA > EtOOH > H2O2 > t-BuOOH. Enzyme Recycling and the Steady State Spectrum of Intermediate II—Fig. 9 shows that the second intermediate, spontaneously formed from compound I when the stoichiometric amounts of the native enzyme and m-Cl PBA reacted, returned to the native enzyme within 2 s. However, if an excess of EtOOH or m-Cl PBA were present, the steady state spectra of intermediate II (similar to spectrum a in Fig. 9A) were stable for more than 20 s, followed by a slow rate of decay that was independent of [ROOH]. The results for the decay of intermediate II in the presence of EtOOH are shown in Fig. 9B. Within experimental error, the observed pseudo-first order rate constant for the decay of intermediate II, \( k_{2,\text{obs}} \), was the same for EtOOH (0.08 ± 0.02 s\(^{-1}\)) and for m-Cl PBA (0.11 ± 0.03 s\(^{-1}\)) (data not shown). The decay rate of intermediate II was not measured for the other hydroperoxides.
compound II. The second order rate constants for compound II formation obtained by the two different kinetic methods were the same for m-Cl PBA and EtOOH. For m-Cl PBA and EtOOH, the formation of compound I is two orders of magnitude faster than the formation of compound II (Tables I and II) so that the two reactions are cleanly separated.

Similar rate constants for compound I and compound II formation were obtained with t-BuOOH (Tables I and II). Thus, the saturation kinetics obtained by the pre-steady state method is a function of two sequential reactions (Fig. 3). The linear kinetics obtained by the transient state method (Fig. 7) is the accurate description for this reaction, showing that the t-BuOOH reaction mechanism is the same as for EtOOH and m-Cl PBA.

For H$_2$O$_2$, the faster phase exhibited saturation kinetics under pre-steady state conditions (Fig. 4), but with pre-formed compound I in the transient state the results were not reproducible. The slower phase of the H$_2$O$_2$ reaction was not reproducible for all experimental conditions. Deleterious effects of H$_2$O$_2$ and/or superoxide appear to be occurring.

For the reaction of PGG$_2$ with compound I under transient state conditions, the saturation kinetics are very similar to those obtained with the pre-steady state experiments (23). If the intramolecular electron transfer mechanism were occurring, then compound I should form the compound I-tyrosyl radical at a rate independent of the concentration of PGG$_2$, which is not the case. The transient state results prove that PGG$_2$ is a reductant of compound I as are other hydroperoxides. The reason that saturation, not linear, kinetics is obtained is that the reaction occurs through the formation of a transient complex between compound I and PGG$_2$. For the previously observed saturation kinetics obtained under pre-steady state conditions, the possibility of complex formation was not taken into consideration, and direct reduction of compound I was erroneously excluded (23).

Tyrosyl Radical Formation—Although our results establish a reaction of compound I with hydroperoxides (Reaction 2), the fact remains that tyrosyl radicals are formed and their mechanism of formation is still unresolved. A plausible explanation is a subsequent reaction of the products of compound I reduction: compound II and the hydroperoxyl radical. If they remain bound, further oxidation-reduction changes could occur, leading to the formation of the compound I-tyrosyl radical and the regeneration of the hydroperoxide (Reaction 5):

$$\text{E-II} + \text{ROO}^- \rightarrow \text{E-I-Tyr} + \text{ROOH}$$

**REACTION 5**

This reaction for tyrosyl radical formation was originally proposed as part of both the branched-chain tyrosyl radical mechanism (23) and the non-chain mechanism (18, 19). Thus, the hydroperoxyl radical could abstract a hydrogen atom from a tyrosyl residue to form the compound I-tyrosyl radical. However, in the branched-chain mechanism, both a unimolecular pathway (no reaction with hydroperoxide) and bimolecular pathway (reaction with a hydroperoxyl radical) for compound I-tyrosyl radical formation were proposed (23). The sum of Reactions 2 and 5 is the following unimolecular reaction:

$$\text{E-I} \rightarrow \text{E-I-Tyr}$$

**REACTION 6**

However, this work excludes the unimolecular pathway in Reaction 6, i.e. direct intramolecular electron transfer from the tyrosyl residue to the porphyrin cation radical.

The compound I-tyrosyl radical cannot be distinguished spectroscopically from compound II since they have the same porphyrin-iron(IV) chromophore. Thus, the measured rate constant $k_2$ cannot be an intrinsic but only an apparent second order rate constant for the reaction of compound I with hydroperoxides. At one extreme, the formation of compound II could be rate-controlling (Reaction 2), as we assumed for the sake of simplicity in the interpretation of the single-exponential kinetic results. At another extreme, the second step, the formation of the compound I-tyrosyl radical in Reaction 5, could be rate-controlling. A combination of the two might explain the biphase kinetics obtained with H$_2$O$_2$.

Further support that compound II is formed before the compound I-tyrosyl radical is obtained from the spectral and kinetic studies on intermediate II (Fig. 9). Intermediate II returned to native enzyme when no excess of hydroperoxide was present, indicating that it is compound II reacting with DDC. When a hydroperoxide was present in high excess, the steady state spectrum of intermediate II was very stable. Intermediate II decayed slowly and independently from the hydroperoxide concentration. This indicates that when DDC was consumed, compound II did not react with neutral hydroperoxide but reacted with the hydroperoxyl radical, causing the formation of the compound I-tyrosyl radical. In the opposite situation, if the hydroperoxides reacted with compound II, the $k_{obs}$ would be a function of the hydroperoxide concentration. The above results are in agreement with EPR studies, which proved that tyrosyl radicals are formed under similar conditions (16, 17, 34–36).

The nature of the steady state species present when an excess of hydroperoxide is added could also be resolved by determining whether a one-electron (for compound II) or a two-electron donor (for compound I-Tyr) converts the species to the native enzyme. We tried to utilize reduced cytochrome c for this purpose. The attempt was unsuccessful because of the instability of the reduced cytochrome c and the high reactivity of the enzyme. The results were not reproducible.

**Very Recent Literature—Knowledge about certain facets of the PGH synthase reaction mechanism is increasing at a rapid rate. Here, we summarize advances made in the current paper and relate them to recent studies.**

The present paper shows for the first time that disappearance of compound I is accelerated by hydroperoxides and that the likely first product is compound II. In addition, the very large rate constant for the reaction of compound I of PGH synthase with phenol was measured and found to be $8.3 \pm 0.2 \times 10^7$ M$^{-1}$ s$^{-1}$ at 6.7°C (Table I).

Recent experiments and pre-steady state models have been published on the reactions of arachidonic and eicosadienoic acids with native enzyme (19). Under pre-steady state conditions, the rate of formation of intermediate II was found to be a function of fatty acid concentration. The rate of reaction of arachidonic acid with compound I ($1.2 \times 10^7$ M$^{-1}$ s$^{-1}$, 30°C; Ref. 19) is significantly slower than the reaction with PGG$_2$ ($1.0 \times 10^6$ M$^{-1}$ s$^{-1}$, 3°C; Table I), which means that arachidonic acid probably could not compete with PGG$_2$ for compound I at higher PGG$_2$ concentrations. For arachidonic acid, the model that fit the experimental data was one in which the products of the compound I-arachidonic acid reaction are compound II and the carbon radical of arachidonic acid. The latter reacts rapidly with molecular oxygen to form the PGG$_2$ radical, which abstracts the phenolic hydrogen atom from Tyr$^{385}$ to form PGG$_2$ and a compound I-tyrosyl radical. The observed second order kinetics with both eicosadienoic and arachidonic acids could be due to fast formation of the corresponding hydroperoxides, which do react with compound I. The experiments with fatty acids were performed under aerobic conditions so that the newly formed hydroperoxides participated in the overall reaction. However, the reduction of compound I by hydroperoxides...
was not included in the pre-steady state model because at that time no data were available indicating that such a reaction occurs. Thus, the pre-steady state models for reactions of fatty acids (19) did not directly prove that the fatty acids react with compound I but did provide evidence against the spontaneous formation of a tyrosyl radical from compound I. The results could not be fit with the branched-chain mechanism. Only future transient state kinetic studies with arachidonic acid and compound I in the absence of oxygen will give a definitive answer as to whether compound I or the compound I-tyrosyl radical formed later in the cycle is the species that initiates the fatty acid oxygenation.

Two papers describe the kinetics of the oxygenase reaction of PGH synthase with cis,cis-eicosa-11,14-dienoic acid (39) and the effect of Trolox C on the reaction (40). A surprising 2:1 ratio occurs in the maximum rate of dienoic acid reaction compared to the maximum rate of molecular oxygen consumption, whereas the expected 1:1 ratio is obtained when the enzyme is soybean lipooxygenase. Trolox C accelerates the PGH synthase-catalyzed reaction at low concentration, and the accelerating effect decreases for higher Trolox C concentration. For all concentrations of Trolox C, from 0 to 250 μM, the 2:1 ratio is maintained. The results are explained in terms of participation of two tyrosyl radicals on the enzyme, one which participates in the reaction cycle (Tyr385) and the other which leads to enzyme inactivation. There is no intrinsic reason why a tyrosyl radical cannot also be found on compound II, and the existence of such a radical is a necessary part of the proposed inactivation mechanism. The effect of Trolox C must be on the inactivation pathways; otherwise, it would change the 2:1 stoichiometry.

In an elegant EPR study, direct proof of arachidonic acid oxidation by a tyrosyl radical was obtained and of coupling between the peroxidase and cyclooxygenase pathways (41). The existence of other tyrosyl radicals is also discussed. Therefore, it is no longer possible to discuss a non-tyrosyl radical reaction mechanism (42).

The Effect of Ferulic Acid—The effect of ferulic acid on the reaction of PGH synthase with arachidonic acid has been re-examined (43). Since there are major disagreements on the ferulic acid results, we discuss them as a separate topic.

The original steady state study of the effect of ferulic acid provided evidence against the branched-chain mechanism for the part of the cyclooxygenase reaction, which was stimulated by ferulic acid (18). Based on the fixed 1:2 stoichiometry between arachidonic and ferulic acids, it was proposed that arachidonic acid reacts with compound I and the peroxidase electron donor ferulic acid with the compound I-tyrosyl radical and compound II, thus preventing enzyme inactivation (18).

In the latest paper, it is concluded that the branched-chain mechanism is correct (43). Reaction mixtures were quenched by addition of cold ether-methanol mixtures and analyzed for PGG2 and PGH2 by thin-layer chromatography. After 10–20 s, product formation remained essentially constant with PGH2 always in excess of PGG2, except for the spontaneous reaction (no added reductant). This indicates that the enzyme is inactivated with respect to cyclooxygenase activity after 10–20 s. Peroxidase activity, tested by adding 15-hydroperoxyeicosatrienoic acid to reaction mixtures containing ferulic acid, also has almost disappeared in the first 20 s.

A major criticism of the earlier study using ferulic acid as a peroxidase substrate is that no correction was applied for the lag between oxygen concentration in bulk solution and in the electrode compartment of the Clark electrode (43). Oxygen diffuses across the electrode membrane via a first order process with a rate constant value typically of the order of 0.25 s⁻¹ (43, 44). The criticism is invalid, because in the earlier study the spontaneous rate of cyclooxygenase activity was subtracted from the total cyclooxygenase activity to get the stimulated rate, and the correction for the diffusion lag period cancels out (18). The experiments on the eicosadienoic acid reaction with PGH synthase and soybean lipooxygenase provide another check on the validity of our measurements of oxygen uptake (19). The correct 1:1 stoichiometry between rate of dienoic acid reaction and rate of oxygen uptake was obtained for the soybean lipooxygenase reaction and provided a calibration for the PGH synthase experiments, performed on the same instrument. Therefore, the oxygen results are not caused by an instrumental artifact. Ferulic acid is highly reactive, even in buffer solution in the presence of light (18), and a large blank correction should have been applied in the most recent work (43).

There are discrepancies in rate constants used for modeling studies (19, 43). Although most of the data in the latest ferulic acid study were obtained under conditions where enzyme inactivation is dominant, no term for inactivation was included in the modeling (43). Partly because of this there is an erroneous assumption of a fixed, large requirement of hydroperoxide for the tightly coupled mechanism, leading to the conclusion that the tightly coupled mechanism cannot fit the experimental data (43); however, it can (19).

It is autoxidation of arachidonic acid, or presence of endogenous oxidant, that initiates the cyclooxygenase reaction. Autoxidation of reactive hydrocarbons is continually occurring and can occur via a non-enzymatic chain reaction. In the horseradish peroxidase reaction with indole-3-acetic acid, there is such a chain reaction (45). Although there is great need for a thorough investigation of the initiation of PGH synthase reactions, it will be very difficult to separate reactions of endogenous and exogenous oxidants.

There are another interesting parallel between the horseradish peroxidase reaction with indole-3-acetic acid and the PGH synthase cyclooxygenase reaction. Some workers believed that the peroxydase reaction with indole-3-acetic acid was initiated by reduction of the Fe(III) of the native enzyme to Fe(I). It definitely does not occur at pH 7 (45). It was thought that the same reduction might be required with PGH synthase, which was disproven by the detection of the oxidized peroxidase-like intermediates of PGH synthase, not reduced enzyme (20). There is no longer any question about compound I and compound II participation in the PGH synthase reactions. The debate is over the precise roles.

Finally, it is claimed that cosubstrate is required for cyclooxygenase activity in the tightly coupled mechanism (43). Our study of the effect of ferulic acid clearly differentiated between a spontaneous cyclooxygenase reaction and a cyclooxygenase reaction stimulated by ferulic acid. There was never any question of the existence of a spontaneous reaction (18).

Summary of Areas of Agreement, Disagreement, and Some Postulates—There now appears to be universal agreement that compounds I and II and a free radical located on Tyr385 of PGH synthase are important participants in the cyclooxygenase reaction. No one can now question the existence of separate binding sites on the enzyme for fatty acid and smaller reductants such as phenols (15). If no fatty acid is present, a conventional peroxidase cycle can be observed in the presence of a hydroperoxide and reducing substrate such as a phenol. Phenols are known to be excellent free radical scavengers. Therefore, conventional small-molecule reductants such as phenols are competitors for reactions of fatty acids with compound I and tyrosyl radicals. Phenols stimulate the cyclooxygenase reaction at lower concentrations and inhibit the stimulated reaction at larger concentrations. The postulate that large concentrations...
of phenols inhibit by competing for the fatty acid binding site is no longer valid; this leaves reaction of phenols with compound I as a valid explanation for inhibition (32). There is no disagreement over the existence of a spontaneous reaction (no added phenol) or of a stimulated reaction (added phenol). However, there is strong disagreement over the relative merits of the tightly coupled mechanism versus the branched-chain mecha

ism and over the relative amounts and roles of PGH₂ and PGG₂. Although evidence is mounting for the role of a second tyrosyl radical, this in not universally accepted.

A plausible explanation for many of the discrepancies in the literature is that many results have been obtained with enzyme that is appreciably inactivated. Enzyme inactivation begins with addition of oxidant to the enzyme and is best minimized by making measurements during the first 5 s of reaction. A chain reaction involving a tyrosyl radical can explain the accumulation of PGG₂, and it is our postulate that this is both a result of, and a pathway to, enzyme inactivation. An appreciable accumulation of PGG₂ in the cold solvent quench experiments could occur in the dead time of the quenching experiments. A question that needs to be addressed is that if PGH₂ is the initial reactant in the synthesis of other prostaglandins and the thromboxanes, why would nature desire an accumulation of highly reactive PGG₂ instead?

Conclusions—This work extends our knowledge about the role of hydroperoxides in the catalytic cycle of PGH synthase. We provide clear evidence that compound I does not spontaneously form the protein (tyrosyl) radical as in compound I of cytochrome c peroxidase. Under conditions where the tyrosyl radicals were usually detected by EPR, i.e., when the hydroperoxides were the only substrates present, we have shown that the natural substrate PGG₂ and a series of structurally diverse hydroperoxides all act as one electron reductant, converting compound I to compound II. Tyrosyl radical formation occurs at a later stage, a unique feature of PGH synthase. The reaction of compound I with PGG₂ is fast enough to be physiologically significant and likely is a part of the catalytic initiation of arachidonic acid oxygenation if the phenols and similar peroxidase electron donors are absent. However, the enzyme species to be a candidate for the initiation of arachidonic acid oxygenation under physiological conditions still is unknown and could be compound I, compound I-tyrosyl radical, or compound II. The PGG₂ reaction with compound I that leads to the tyrosyl radical formation and subsequent chain reaction with arachidonic acid, however, may be irrelevant for the PGH₂ formation if peroxidase electron donors are present. It may be a feedback inactivation reaction in the absence of peroxidase electron donors.

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J. Biol. Chem. 1996, 271:2048-2056.
doi: 10.1074/jbc.271.4.2048

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