Intimate Relation between Cyclooxygenase and Peroxidase Acivities of Prostaglandin H Synthase. Peroxidase Reaction of Ferulic Acid and Its Influence on the Reaction of Arachidonic Acid

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Received November 11, 1993; Revised Manuscript Received March 17, 1994®

ABSTRACT: The oxidation of ferulic acid by hydrogen peroxide catalyzed by prostaglandin H synthase follows a modified ping-pong irreversible mechanism, as is the case for classical peroxidases. The rate constant for the reaction of prostaglandin H synthase with hydrogen peroxide, determined from steady-state results, is $(1.31 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, and for the reaction of prostaglandin H synthase–compound II with ferulic acid it is $(5.5 \pm 0.3) \times 10^6$ M^{-1} s^{-1}. Cyclooxygenase and peroxidase functions of prostaglandin H synthase were studied by comparing the initial rates of reaction of the cyclooxygenase substrate, arachidonic acid, and a peroxidase reducing substrate, ferulic acid, in mixtures of the two substrates. For both an equimolar ratio of arachidonic and ferulic acids and ferulic acid in excess of arachidonic acid a stimulation of the cyclooxygenase reaction is observed. The concentration of ferulic acid necessary to produce 50% stimulation of 0.2 mM arachidonic acid oxidation is 0.14 ± 0.02 mM. A striking feature of our results is that prostaglandin H synthase catalyzes oxidation of the two substrates in a constant and fixed molar ratio of ferulic acid to arachidonic acid of 2:1, despite widely different starting concentrations. If arachidonic acid is in excess of ferulic acid, enzyme inactivation occurs. The results can be explained by an interconnected cyclooxygenase–peroxidase unbranched free radical mechanism in which arachidonic acid reacts with either the ferryl oxygen or the porphyrin π-cation radical part of a conventional peroxidase compound I (a Fe^{IV}=O porphyrin π-cation radical) and ferulic acid reacts with compound II (Fe^{IV}=O). The ferulic acid also acts as a hydrogen atom donor to the hydroperoxyl radical, $PGG_2^{\bullet}$, or alternatively to the tyrosyl radical providing protection from inactivation. Our data provide strong evidence against a cyclooxygenase branched chain free radical mechanism, in which a cyclooxygenase cycle, once started, can operate with no connection to the peroxidase cycle.

The primary enzyme of prostaglandin and thromboxane biosynthesis is a microsomal cyclooxygenase–peroxidase, prostaglandin endoperoxide synthase, commonly called prostaglandin H synthase (PGH synthase).[1] The enzyme is a homodimer having one catalytically active heme per subunit (Roth et al., 1981; Kulmacz & Lands, 1984; Picot & Garavito, 1989).

According to the ping-pong irreversible reaction mechanism established previously for the monophenol *p*-coumaric acid (Bakovic & Dunford, 1993), the peroxidase cycle for the oxidation of phenol by prostaglandin H synthase and hydrogen peroxide is as follows:

$$\text{PGH synthase} + H_2O_2 \xrightarrow{k_1} \text{compound I} \quad (1)$$

$$\text{compound I} + \text{HOPh} \xrightarrow{k_2} \text{compound II} + \text{OPh}^{\bullet} \quad (2)$$

$$\text{compound II} + \text{HOPh} \xrightarrow{k_3} \text{PGH synthase} + \text{OPh}^{\bullet} \quad (3)$$

This is the classical cycle for all peroxidases (Dunford, 1991). In the present study we establish the same reaction mechanism for the phenol ferulic acid, chosen for its high reactivity and because its reaction is readily followed spectrophotometrically. The establishment of the reaction mechanism of ferulic acid with PGH synthase in the absence of arachidonic acid is essential before examining the effect of ferulic acid on the reaction of arachidonic acid. In the cyclooxygenase reaction two molecules of oxygen are inserted into arachidonic acid, yielding a transient endoperoxide hydroperoxide, prostaglandin G_2 (cyclooxygenase function):

$$AA + 2O_2 \xrightarrow{\text{PGH synthase}} PGG_2 \quad (4)$$

The resultant PGG_2 is reduced to its alcohol analogue, prostaglandin H_2 (peroxidase function) (Miyamoto et al., 1976; van der Oudera et al., 1977; Ohki et al., 1979; Pagels et al., 1983). The mutual relationship of the two enzyme functions, cyclooxygenase and peroxidase, and the overall mechanism are still under investigation and are of considerable interest (Smith & Marnett, 1991; Smith et al., 1992).

Here we examine two mechanisms which it has been claimed account for the experimental observations when both arachidonic acid and a reducing substrate are present. The first is

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® Abstract published in *Advance ACS Abstracts*, May 1, 1994.

[1] Abbreviations: PGH synthase, prostaglandin endoperoxide synthase (EC 1.14.99.1); Cpd-I'-Tyr•, tyrosyl radical form of compound I of PGH synthase formed by internal electron transfer; HAA, arachidonic acid; AA•, free radical derived by hydrogen atom abstraction of the 13-*pro-S* hydrogen atom from AA; PGG_2, hydroperoxide prostaglandin G_2; $PGG_2^{\bullet}$, hydroperoxyl radical of PGG_2; PGH_2, prostaglandin H_2, the alcohol obtained by reduction of PGG_2; HOPh, phenol reducing substrate, in this study ferulic acid; OPh•, free radical derived by hydrogen atom abstraction from ferulic acid; DDC, diethyldithiocarbamate; ϵ, molar absorptivity; FA, ferulic acid; FApr, product of ferulic acid oxidation; PGHS, prostaglandin H synthase; $\Delta\epsilon_{FA}$, difference in molar absorptivity between ferulic acid and its oxidation product.

0006-2960/94/0433-6475$04.50/0

the tyrosyl radical chain mechanism (Dietz et al., 1988; Smith et al., 1992):

Initiation

$$\text{PGH synthase} + PGG_2 \rightarrow \text{compound I} + PGH_2 \quad (5)$$

$$\text{compound I} \rightarrow \text{Cpd-I'-Tyr}^{\bullet} \quad (6)$$

Propagation

$$\text{Cpd-I'-Tyr}^{\bullet} + HAA \rightarrow \text{compound II} + AA^{\bullet} \quad (7)$$

$$2O_2 + AA^{\bullet} \rightarrow PGG_2^{\bullet} \quad (8)$$

$$\text{compound II} + PGG_2^{\bullet} \rightarrow \text{Cpd-I'-Tyr}^{\bullet} + PGG_2 \quad (9)$$

Termination

$$\text{compound I} + HOPh \rightarrow \text{compound II} + OPh^{\bullet} \quad (10)$$

$$\text{compound II} + HOPh \rightarrow \text{PGH synthase} + OPh^{\bullet} \quad (11)$$

According to the above hypothesis, after initiation by formation of compound I (eq 5), which converts spontaneously to a tyrosyl radical form containing the same total number of oxidizing equivalents (eq 6), the tyrosyl radical can be regenerated many times (eqs 7–9). The phenol radicals $OPh^{\bullet}$, formed in eqs 10 and 11, are not scavenged by molecular oxygen; by reacting with each other, they would be chain terminators in the above mechanism (Hewson & Dunford, 1976). No connection between the rates of arachidonic acid and ferulic acid disappearance would be expected for different starting concentrations of the two substrates. Furthermore, the tyrosyl radical chain mechanism would be inhibited by added phenols in two ways: phenols, by competing for compound I, would inhibit the initiation steps and, by reacting with compound II, would also interfere with the propagation steps. Thus with increasing phenol concentration the ratio of phenol/arachidonic acid reacted would also be expected to increase.

The alternative mechanism is one which does not involve a branched chain reaction but rather a normal peroxidatic reaction cycle in which one of the steps is abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid by the typical peroxidase compound I, a Fe^{IV}=O porphyrin π-cation radical (Hsuanyu & Dunford, 1992):

$$\text{PGH synthase} + PGG_2 \rightarrow \text{compound I} + PGH_2 \quad (5)$$

$$\text{compound I} + HAA \rightarrow \text{compound II} + AA^{\bullet} \quad (12)$$

$$2O_2 + AA^{\bullet} \rightarrow PGG_2^{\bullet} \quad (8)$$

$$HOPh + PGG_2^{\bullet} \rightarrow PGG_2 + OPh^{\bullet} \quad (13)$$

$$\text{compound II} + HOPh \rightarrow \text{PGH synthase} + OPh^{\bullet} \quad (11)$$

The sum of these reactions is

$$HAA + 2HOPh + 2O_2 \rightarrow PGH_2 + 2OPh^{\bullet} \quad (14)$$

The above peroxidase mechanism (arachidonic acid reacting with the classical compound I and the phenol, ferulic acid, reacting with compound II) leads to the prediction of a fixed 2:1 ratio of phenol/arachidonic acid reacted, and this is what we have observed and report upon in this paper.

MATERIALS AND METHODS

PGH synthase was purified from ram seminal vesicle microsomes using the modification of MacDonald and Dunford (1989) of the procedure of Marnett et al. (1984). The isolated enzyme was a 40/60 mixture of holo- and apoenzyme having a specific cyclooxygenase activity of 37 μM arachidonic acid (mg of protein)$^{-1}$ min^{-1} in the presence of 1 μM hematin and 1 mM phenol. Enzyme concentrations were assayed spectrophotometrically using an ϵ_{PGHS} of 123 mM^{-1} cm^{-1} at 410 nm (Kulmacz & Lands, 1984), and protein content, by the Bio-Rad protein assay using bovine serum albumin as a standard.

Arachidonic and ferulic acids and hematin were obtained from Sigma, and hydrogen peroxide was from Fisher. Arachidonic acid was dissolved in deaerated 98% ethanol solution and kept in small portions under nitrogen at −70 °C. Ferulic acid was recrystallized from water. The concentration of hydrogen peroxide was determined spectrophotometrically using $\epsilon_{H_2O_2}$ = 43.6 M^{-1} cm^{-1} at 240 nm (Beers & Sizer, 1952).

Curve fittings and calculations on all of our results were made using a nonlinear regression data analysis program by Enzfitter, Elsevier-Biosoft.

Oxidation of Ferulic Acid by Hydrogen Peroxide Catalyzed by PGH Synthase. A Union Giken RA-601 stopped-flow apparatus was used for the initial rate measurements. The changes in absorbance at 310 nm due to the reaction of ferulic acid with prostaglandin H synthase and hydrogen peroxide as a function of time were measured using constant 21.3 or 25.7 nM enzyme and varying and high concentrations of hydrogen peroxide. Kinetic traces, repeated at least eight times for each set of conditions, were obtained. The initial rates of disappearance of ferulic acid were determined on a time scale of 0.5–1.0 s with experimental errors which were less than 8%.

Oxidation of Ferulic Acid by Arachidonic Acid Catalyzed by Prostaglandin H Synthase. The rate of ferulic acid oxidation was determined using stopped-flow measurements as described above. One reservoir was used for arachidonic acid solutions, and the other, for enzyme and ferulic acid, with or without 1 μM hematin, all in 0.1 M phosphate buffer of pH 8.0. The enzyme and ferulic acid mixtures, with or without added hematin, were preincubated for 3 min at 30 °C. The initial rates of ferulic acid disappearance were determined at 310 nm from kinetic traces that were always linear for times up to 2 s. To avoid the effect of autooxidation of arachidonic acid, repetitive traces were obtained within 5 min of preparation of the arachidonic acid solutions. For each reaction mixture placed in the stopped-flow apparatus at least seven repetitive traces were obtained, and duplicate or triplicate reaction mixtures were used.

Oxygen Uptake by Arachidonic Acid Catalyzed by PGH Synthase and Stimulated by Ferulic Acid. The rate of arachidonic acid oxidation catalyzed by PGH synthase was determined by monitoring the rate of oxygen consumption as described previously (Bakovic & Dunford, 1993). Triplicate experiments were performed in the absence and presence of added hematin. The incubation mixture without added hematin contained 0.1 M phosphate buffer of pH 8.0, 50 nM enzyme, and arachidonic acid and ferulic acid in varying concentrations. Cyclooxygenase reactions in the absence of ferulic acid but under otherwise identical conditions were also measured. The concentration of dissolved oxygen was 230

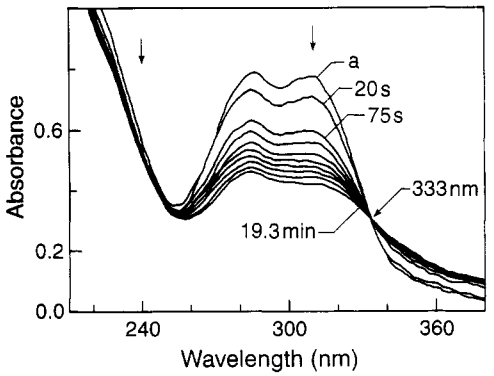

FIGURE 1: Oxidation of ferulic acid with hydrogen peroxide catalyzed by PGH synthase: changes in the absorption spectrum of ferulic acid (48.5 μM) during its oxidation with 0.265 mM H_2O_2 and 53.9 nM PGH synthase. Scans were taken over 19.3 min. Spectrum a is a pure ferulic acid spectrum taken in a separate experiment.

μM at 30 °C (Robinson & Cooper, 1970). The rate of arachidonic acid disappearance was determined assuming that 2 mol of oxygen was consumed per mol of arachidonic acid. There is no oxygen uptake by phenolic radicals (Hewson & Dunford, 1976).

RESULTS

Spectral Behavior of Ferulic Acid in a PGH Synthase Peroxidase Reaction. Spectra of the reaction of ferulic acid with hydrogen peroxide, catalyzed by PGH synthase, were recorded using a Cary 219 spectrophotometer in the 220–380-nm region where ferulic acid absorbs strongly. Maxima are observed at 286 and 310 nm in a phosphate buffer, pH 8.0 (Figure 1). Measurements at 310 nm were used for the kinetic steady-state measurements because a significant drop in absorbance occurs when ferulic acid is oxidized. Furthermore, there is no significant interference in absorption from hydrogen peroxide or a steady-state concentration of enzyme at 310 nm.

Beer's law was obeyed over the range 5–150 μM ferulic acid in 0.1 M phosphate buffer, pH 8.0, at room temperature, from which we determined ϵ_{FA} at 310 nm to be 14.84 ± 0.01 mM^{-1} cm^{-1}. Reaction of ferulic acid with excess hydrogen peroxide, catalyzed by PGH synthase, was incomplete. Upon addition of more PGH synthase the reaction could be driven to completion. For completed reactions the absorbances of the ferulic acid product at 310 nm were determined for a series of ferulic acid concentrations. The product of ferulic acid oxidation has an ϵ_{FApr} of 7.50 ± 0.15 mM^{-1} cm^{-1}. The molar absorptivity coefficient for pure ferulic acid was then corrected for this value, and the value $\Delta\epsilon_{FA}$ = 7.34 mM^{-1} cm^{-1} was obtained. The latter value was used in the calculations of the initial rate of disappearance of ferulic acid.

Ferulic acid by itself in 0.1 M phosphate buffer, pH 8.0, at 30 °C decays spontaneously when exposed to the 310-nm light of the stopped-flow apparatus. The overall rates of ferulic acid disappearance obtained in the presence of the enzyme were corrected appropriately using duplicate blank experiments (enzyme and ferulic acid but no arachidonic acid or hydrogen peroxide).

In the presence of excess hydrogen peroxide and a catalytic amount of PGH synthase, ferulic acid is rapidly oxidized. A single product is formed from ferulic acid as shown by an isosbestic point at 333 nm (Figure 1). The reaction shown is 85% complete. A subsequent addition of PGH synthase caused an additional drop in absorbance (data not shown). The enzyme and hydrogen peroxide were added once more,

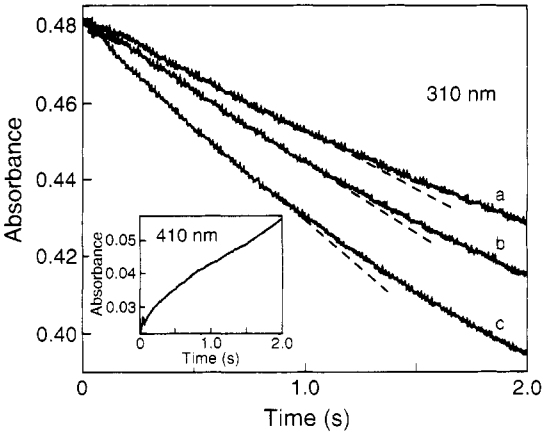

FIGURE 2: Effect of hydrogen peroxide concentration on the initial rate of ferulic acid disappearance in the PGH synthase catalyzed peroxidatic reaction: kinetic traces of ferulic acid (32.5 μM) at 310 nm after its reaction with 25.7 nM enzyme and (a) 0.365, (b) 0.908, and (c) 1.29 mM H_2O_2 in 0.1 M phosphate buffer, pH 8.0, at 25 ± 0.5 °C. Inset: Rate of change of absorbance at 410 nm (the maximum absorbance for the native enzyme) caused by conversion of prostaglandin H synthase–compound II back to the native enzyme by its reaction with ferulic acid. The reaction mixture contained 32.5 μM ferulic acid, 0.5 mM H_2O_2, and 0.15 μM enzyme. Other conditions were the same as in the 310-nm traces.

but did not cause further changes in the product spectrum. Thus the molar absorptivity of the product of ferulic acid oxidation was obtained.

Steady-State Kinetics of the Reaction of Ferulic Acid with Hydrogen Peroxide, Catalyzed by Prostaglandin H Synthase. In order to obtain maximum sensitivity in rate measurements, the reaction conditions were first optimized. From the molar absorptivity measurements at 310 nm, we found that the ferulic acid absorption can be measured in the 5–150 μM range. We also found that a PGH synthase concentration in the 20–50 nM range is optimal to give linear responses in initial rates.

Representative examples of the initial rate measurements are presented in Figure 2. The kinetic traces at 310 nm are linear below 1.0 s, and the rates of ferulic acid disappearance were found to be a linear function of $[H_2O_2]$ for five different fixed concentrations of ferulic acid. The initial rates were calculated from the slopes of the linear parts of the traces, in the 0.5–1.0 s time scale, by applying Beer's law and using $\Delta\epsilon_{FA}$. Data are plotted, for a 1:2 molar ratio of hydrogen peroxide and ferulic acid, as $v/[E]_{tot}$ versus $[H_2O_2]$ (Figure 3). At higher hydrogen peroxide concentrations the reaction rate increased, but the departure from linearity occurred in a shorter time (Figure 2).

Our early work (Lambeir et al., 1985) demonstrated that the Soret spectrum of the native enzyme, with a maximum at 410 nm, decreases upon reaction with hydroperoxide, indicating compound I formation as in other peroxidases. PGH synthase–compound I has reduced absorptivity at 410 nm and an isosbestic point with the native enzyme at 426 nm. PGH synthase–compound II has a red-shifted spectrum with a maximum at 420 nm. The only enzyme intermediate observed under our conditions is PGH synthase–compound II. The inset to Figure 2 is a kinetic trace at 410 nm (the absorption maximum for the native enzyme) showing that PGH synthase–compound II slowly converts to the native enzyme when 32.5 μM ferulic acid, 0.5 mM hydrogen peroxide, and 0.15 μM enzyme are used. A similar kinetic trace was obtained at 426 nm, the isosbestic point between native enzyme and PGH synthase–compound I. Furthermore, at lower [ferulic acid] and higher $[H_2O_2]$, the PGH synthase–compound II conversion is slower (data not shown).

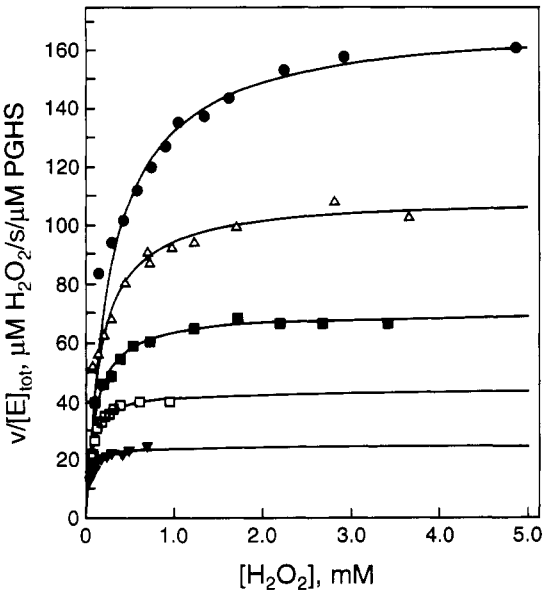

FIGURE 3: Steady-state initial rates at unit total enzyme concentations of the reduction of the hydrogen peroxide obtained for the series of ferulic acid concentrations. FA concentrations were 6.8 (▼), 11.9 (□), 19.8 (■), 31.9 (△), and 57.0 (●) μM.; PGH synthase (21.3 nM) having a specific activity of 34.5 μM AA (mg of protein)$^{-1}$ min^{-1} was used for these experiments. Other conditions were the same as in Figure 2.

The rate expression corresponding to eqs 1–3 is

$$-d[H_2O_2]/dt = -(0.5)d[\text{ferulic acid}]/dt = (A[H_2O_2][E]_{tot})/(B + [H_2O_2]) \quad (15)$$

The parameters A and B are

$$A = ((0.5)k_2k_3[\text{ferulic acid}])/(k_2 + k_3) \quad (16)$$

$$B = (k_2k_3[\text{ferulic acid}])/k_1(k_2 + k_3) \quad (17)$$

Consequently,

$$2A/B = k_1 \quad (18)$$

The third step, eq 3, is a rate-controlling step under our experimental conditions, and eq 16 can be simplified to eq 19:

$$A = k_3[\text{ferulic acid}]/2 \quad (19)$$

The experimental values in Figure 3 are shown for the initial rates of the oxidation of ferulic acid with PGH synthase and hydrogen peroxide and the theoretical curve fits to rectangular hyperbolae. Values for the parameters A and B (eqs 16 and 17), summarized in Table 1, were used for linear curve fittings, applying eqs 18 and 19 from the above mechanism. From eq 19 the rate constant for the reaction of PGH synthase–compound II with ferulic acid, k_3, is (5.5 ± 0.3) × 10^6 M^{-1} s^{-1}.

According to eq 18, the linear dependence of the $2A$ parameter versus the B parameter (numerical values from Table 1) is obtained, with the slope of k_1 = (1.31 ± 0.03) × 10^6 M^{-1} s^{-1} and an intercept not significantly different from zero.

Stimulation of Prostaglandin H Synthase Cyclooxygenase Activity by Ferulic Acid. Ferulic acid stimulates the oxidation of arachidonic acid catalyzed by PGH synthase. A concentration of 0.14 ± 0.02 mM ferulic acid resulted in 50% stimulation when 0.2 mM arachidonic acid and 20 nM enzyme were used. The measurements were made for a series of ferulic acid concentrations, but not above 1.5 mM ferulic acid because of its limited solubility in 0.1 M phosphate buffer, pH 8.0.

Table 1: Steady-State Kinetic Parameters for the Reaction of Ferulic Acid and Hydrogen Peroxide Catalyzed by Prostaglandin H Synthase[a]

ferulic acid (μM)	$2A$ parameter[b] (s^{-1})	B parameter[b] (mM)
6.8	48.7 ± 0.4[c]	0.035 ± 0.002
11.9	87.0 ± 0.8	0.057 ± 0.003
19.8	139 ± 1	0.10 ± 0.01
31.9	218 ± 2	0.16 ± 0.01
57.0	329 ± 3	0.25 ± 0.02

[a] Data were obtained by plotting the initial rate of hydrogen peroxide disappearance per unit of enzyme concentration ($v/[E]_{tot}$) against $[H_2O_2]$ at fixed concentrations of ferulic acid. All measurements were performed in 0.1 M phosphate buffer, pH 8.0, at 25 ± 0.5 °C. [b] Parameters A and B were obtained after fitting the experimental data to rectangular hyperbolae. [c] Standard deviation.

Under the same experimental conditions except for the presence of enzyme, hydrogen peroxide initiated a non-enzymatic oxidation of arachidonic acid that included oxygen consumption. Ferulic acid efficiently inhibited the non-enzymatic arachidonic acid oxidation, behaving as a classical antioxidant (data not shown).

Cyclooxygenase–Peroxidase Reaction with Equimolar Amounts of Arachidonic and Ferulic Acids. The kinetics of arachidonic acid oxidation (measured with an oxygen electrode) and of ferulic acid oxidation (measured spectrophotometrically on the stopped-flow apparatus) were examined under first-order conditions for a molar ratio of arachidonic acid/ferulic acid of 1:1, allowing maximum competition between the two substrates for reaction with the enzyme. The effect of added hematin on both reactions was also examined.

There is a significant rate of oxygen uptake (arachidonic acid disappearance) in the absence of the ferulic acid reducing substrate, and this was determined independently. An example of the magnitude of stimulation of the cyclooxygenase reaction by ferulic acid, i.e., the difference in the initial rates of arachidonic acid disappearance, $-d\Delta[\text{arachidonic acid}]/dt$, in the presence and absence of ferulic acid, is shown in Figure 4A. The corresponding rate of disappearance of ferulic acid is shown in Figure 4B, on the same time scale as for the oxygen uptake experiments for direct visual comparison and on a shorter time scale which was used for quantitative rate measurements.

Stimulation of the cyclooxygenase reaction by ferulic acid was observed for micromolar ratios of arachidonic acid/ferulic acid of 20:20, 50:50, and 100:100 with and without added hematin. Tables 2 and 3 show the results along with the corrections for spontaneous decomposition of ferulic acid. Rates of reaction increased with increasing concentrations of substrates and also upon hematin addition, but remarkably, the ratio of the ferulic acid disappearance to the stimulated rate of arachidonic acid disappearance remained constant with a value of 2.

$$\frac{-d[\text{ferulic acid}]/dt}{-d[\text{arachidonic acid}]/dt} = \frac{d[\text{ferulic acid}]}{d[\text{arachidonic acid}]} = 2 \quad (20)$$

Cyclooxygenase–Peroxidase Reactions with Ferulic Acid in Excess Arachidonic Acid. Two sets of experiments were performed in this series: (i) arachidonic acid concentration was kept constant, and ferulic acid concentration was varied; and (ii) ferulic acid concentration was kept constant, and arachidonic acid concentration was varied. The first set of

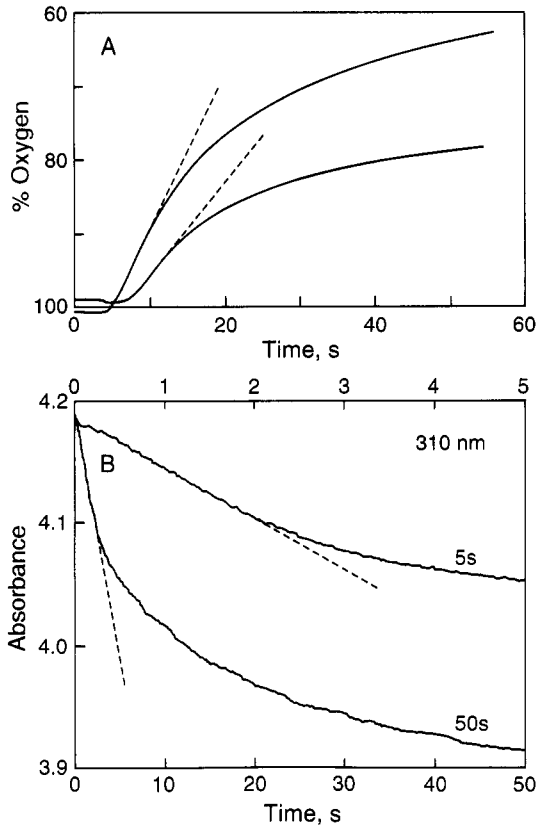

FIGURE 4: Comparison of PGH synthase cyclooxygenase and peroxidase kinetics. (A) Oxygen uptake observed upon incubation of PGH synthase with arachidonic acid in the presence or absence of ferulic acid. Upper curve: 50 μM arachidonic acid, 50 μM ferulic acid, 35 nM PGH synthase, and 1 μM hematin in 0.1 M phosphate buffer, pH 8.0, at 30.0 ± 0.5 °C; lower curve: same conditions except no ferulic acid was added. (B) Kinetic traces at 310 nm of the oxidation of ferulic acid for 5 and 50 s. Arachidonic acid absorbance at 310 nm is unsignificant. Reaction conditions were as in panel A (upper curve).

experiments were performed in the presence and absence of added hematin; the second set, only in the presence of added hematin.

In the first set of experiments the rates of disappearance of the two substrates were monitored for ferulic acid concentrations of 50, 100, and 200 μM and an arachidonic acid concentration of 20 μM ([ferulic acid]/[arachidonic acid] ratios of 2.5:1, 5:1, and 10:1 in Tables 2 and 3). The 2-fold increase in ferulic acid concentration from 50 to 100 μM is accompanied by a parallel increase in both the peroxidase reaction and the stimulation of the cyclooxygenase reaction (1.5- and 1.4-fold, respectively). When the ferulic acid concentration was increased from 100 to 200 μM, no change was observed in the rates of both the peroxidase and cyclooxygenase reactions for the holo-/apoenzyme, whereas inhibition of both activities occurred for the reconstructed enzyme. Maximum rates for both the peroxidase reaction and the stimulated cyclooxygenase reaction are attained when the [ferulic acid]/[arachidonic acid] ratio is 5:1. Even though marked changes were observed in the rates of reaction as a function of the concentration of ferulic acid, the ratio of the rate of ferulic acid disappearance to the stimulated rate of arachidonic acid disappearance remained constant with a value of 2 as in the 1:1 series of experiments.

In the second set of experiments, in which arachidonic acid concentration was varied and ferulic acid concentration was kept constant ([ferulic acid]/[arachidonic acid] ratios of 2:1, 4:1, and 20:1; Table 3), the rates of the peroxidase reaction and the stimulation of the cyclooxygenase reaction were functions of arachidonic acid concentration, but again, the ratios of the two rates were constant with a value of 2.

Reaction of PGH Synthase with Arachidonic Acid in Excess Ferulic Acid. The results obtained with arachidonic acid in excess are considerably different from those described above. There was considerable ferulic acid oxidation but no significant stimulation of arachidonic acid oxidation in all experiments except one (Tables 2 and 3). The one exception was when ferulic acid and arachidonic acid, 20:80 μM, were reacted with reconstituted enzyme (Table 3). The lack of stimulation is more apparent at lower concentrations of holo-/apoenzyme (Table 2) than at higher concentrations of reconstructed enzyme (Table 3). For 1:4 and 1:10 ratios of ferulic acid to arachidonic acid using PGH synthase as isolated, as well as for a 1:10 ratio of ferulic acid to arachidonic acid using the reconstructed enzyme, there is no significant stimulation of arachidonic acid disappearance. Consequently, d[ferulic acid]/dΔ[arachidonic acid] ratios became significantly larger than 2. The results are opposite what might be anticipated. Since the cyclooxygenase reaction involves arachidonic acid, with excess arachidonic acid a faster rate of its disappearance might be expected, yielding a d[ferulic acid]/dΔ[arachidonic acid] ratio lower than 2. It appears that at higher arachidonic acid concentrations enzyme inactivation takes place.

DISCUSSION

PGH Synthase, Hydrogen Peroxide, and Ferulic Acid. Ferulic acid is oxidized with prostaglandin H synthase higher oxidation states through two hydrogen atom abstractions yielding free radicals that combine non-enzymatically. Under steady-state conditions, the only enzyme intermediate detected is compound II. The inset to Figure 2 shows a kinetic trace for the conversion of PGH synthase–compound II back to the native enzyme. The initial burst in the kinetic traces indicates that yet another intermediate, namely, PGH synthase–compound I, is formed in the pre-steady-state phase. The results of the initial rate measurements reveal that ferulic acid is oxidized by PGH synthase higher oxidation state intermediates through the same irreversible ping-pong mechanism as for p-coumaric acid (Bakovic & Dunford, 1993), as is typical for all peroxidases.

Arachidonic Acid, Ferulic Acid, and Hydrogen Peroxide. Our experiments show that ferulic acid prevents a non-enzymatic oxidation of arachidonic acid by hydrogen peroxide. In the absence of enzyme, oxygen consumption ceased in the presence of ferulic acid, indicating inhibition of arachidonic acid oxidation. Ferulic acid probably acts as a classical chain-breaking antioxidant by efficiently removing peroxide radicals initially formed from arachidonic acid (Burton & Ingold, 1989).

Effect of Ferulic Acid on the PGH Synthase–Arachidonic Acid Reaction. In marked contrast to the non-enzymatic reaction, our results show that ferulic acid stimulates the enzyme-catalyzed arachidonic acid oxidation. The concentrations necessary for 50% stimulation of 0.2 mM arachidonic acid oxidation are in the order ferulic acid > *p*-coumaric acid (Bakovic & Dunford, 1993) in agreement with these compounds' reduction potentials and the rate constants for their reactions with PGH synthase compound II. The hyperbolic curves obtained by a plot of O_2 consumption versus [ferulic acid] (data not shown) suggest that ferulic acid is playing an important role in the enzymatic cycle involving arachidonic acid.

Direct Comparison of the Cyclooxygenase and Peroxidase Reactions. In order to acquire a better understanding of what

Table 2: Simultaneous Oxidation of Arachidonic and Ferulic Acids Catalyzed by a 40:60 Mixture of Holo- and Apoprostaglandin H Synthase: Relation between the Rate of Ferulic Acid (FA) Disappearance and the Magnitude of Its Stimulation of Arachidonic Acid (AA) Disappearance[a]

		stopped-flow measurements[b]			oxygen uptake measurements[c]			
[FA]:[AA]	μM FA:μM AA	$slope_{tot}$ (ΔA s^{-1})	$slope_{bl}$ (ΔA s^{-1})	$-d[FA]/dt$ (μM s^{-1})	$slope_{st}$ (s^{-1})	$slope_{sp}$ (s^{-1})	$-d\Delta[AA]/dt$ (μM s^{-1})	$d[FA]/d\Delta[AA]$
		A. Equal Amounts of Arachidonic and Ferulic Acids						
1:1	20:20	0.0149	0.0090	0.804	0.0041	0.0025	0.368	2.2
1:1	50:50	0.0367	0.0290	1.05	0.0050	0.0030	0.460	2.3
1:1	100:100	0.0510	0.0370	1.91	0.0056	0.0025	0.713	2.7
		B. Arachidonic Acid Concentration Constant; Ferulic Acid in Excess						
2.5:1	50:20	0.0386	0.0290	1.31	0.0052	0.0025	0.621	2.1
5:1	100:20	0.0520	0.0370	2.04	0.0063	0.0025	0.874	2.3
10:1	200:20	0.0499	0.0360	1.90	0.0067	0.0025	0.966	2.0
		C. Ferulic Acid Concentration Constant; Arachidonic Acid in Excess						
1:4	20:80	0.0200	0.0150	0.681	0.0033	0.0030	0.069	9.9
1:10	20:200	0.0245	0.0150	1.29	0.0031	0.0031	NS[d]	
1:2	50:100	0.0392	0.0290	1.40	0.0044	0.0025	0.437	3.2
1:4	50:200	0.0426	0.0290	1.85	0.0033	0.0033	NS	

[a] 50 nM PGH synthase. [b] $slope_{tot}$ represents the initial change in the absorbance at 310 nm for the catalyzed degradation of ferulic acid in the presence of arachidonic acid. $slope_{bl}$ is the initial slope for the spontaneous decay of ferulic acid. The difference between the two slopes divided by $\Delta\epsilon_{FA}$ gives the rate of ferulic acid disappearance ($-d[FA]/dt$ in μM s^{-1}) for the catalyzed reaction. [c] $slope_{st}$ and $slope_{sp}$ are one-half the initial slopes of the oxygen consumption curves obtained for the catalyzed oxygenation of arachidonic acid in the absence and presence of ferulic acid; the difference between the two slopes multiplied by the amount of dissolved oxygen (230 μM) results in the rates, $d\Delta[AA]/dt$ in μM s^{-1}. [d] No stimulation of oxygen uptake by ferulic acid.

Table 3: Simultaneous Oxidation of Arachidonic Acid and Ferulic Acid Catalyzed by Prostaglandin H Synthase with Added Hematin: Relation between the Rates of Ferulic Acid (FA) Disappearance and the Magnitude of Its Stimulation of Arachidonic Acid (AA) Disappearance[a]

		stopped-flow measurements[b]			oxygen uptake measurements[c]			
[FA]:[AA]	μM FA:μM AA	$slope_{tot}$ (ΔA s^{-1})	$slope_{bl}$ (ΔA s^{-1})	$-d[FA]/dt$ (μM s^{-1})	$slope_{st}$ (s^{-1})	$slope_{sp}$ (s^{-1})	$d\Delta[AA]/dt$ (μM s^{-1})	$d[FA]/d\Delta[AA]$
		A. Equal Amounts of Arachidonic and Ferulic Acids						
1:1	20:20	0.0180	0.0090	1.23	0.0065	0.0040	0.575	2.1
1:1	50:50	0.0400	0.0260	1.91	0.0095	0.0059	0.828	2.3
1:1	100:100	0.0737	0.0348	5.25	0.0135	0.0038	2.23	2.4
		B. Ferulic Acid Concentration Constant and in Excess						
20:1	200:10	0.0520	0.0360	2.18	0.0081	0.0035	1.05	2.1
4:1	200:50	0.0620	0.0360	3.54	0.0150	0.0059	2.09	1.7
2:1	200:100	0.0640	0.0360	3.81	0.0140	0.0040	2.30	1.7
		C. Arachidonic Acid Concentration Constant; Ferulic Acid in Excess						
2.5:1	50:20	0.0527	0.0290	3.23	0.0102	0.0040	1.43	2.3
5:1	100:20	0.0723	0.0370	4.81	0.0124	0.0040	1.93	2.5
10:1	200:20	0.0630	0.0360	3.68	0.0112	0.0040	1.66	2.2
		D. Ferulic Acid Concentration Constant; Arachidonic Acid in Excess						
1:4	20:80	0.0128	0.0030	1.34	0.0055	0.0030	0.575	2.3
1:10	20:200	0.0211	0.0030	2.46	0.0050	0.0025	0.575	4.3

[a] A, C, and D: 35 nM PGH synthase. B: 50 nM PGH synthase. [b] Symbols, units, and corrections made for the spontaneous decay of ferulic acid are the same as in Table 2. [c] Symbols, units, and calculations are the same as in Table 2.

features make a PGH synthase cyclooxygenase reaction similar to or different from a PGH synthase peroxidase reaction, measurements were made, for identical reaction mixtures consisting of PGH synthase, arachidonic acid, and ferulic acid, of the initial rates of disappearance of the substrates. For the cyclooxygenase reaction (reaction of arachidonic acid) the rate of oxygen consumption was measured using an oxygen electrode. For the peroxidase reaction (disappearance of ferulic acid) we utilized the previously developed system of stopped-flow steady-state studies for accurate measurements of initial rates (Bakovic & Dunford, 1993). The minimum times for measurements on the two instruments are somewhat different, but direct comparisons for identical time scales were possible and were made. Free radicals derived from ferulic acid, like those from other monophenols, do not react with molecular oxygen, so there is no ambiguity in the measurements. Furthermore, neither arachidonic acid nor its oxidation products interfere with ferulic acid absorbance at 310 nm.

In the present study the ratio of peroxidase to cyclooxygenase activity was evaluated for series of arachidonic acid and ferulic acid concentrations for two PGH synthase preparations, one using the enzyme as isolated and the other using enzyme reconstructed with added hematin. When the overall rate of arachidonic acid disappearance, corrected for the spontaneous rate of arachidonic acid disappearance, was related to the corresponding rates of ferulic acid disappearance, constant ratios were demonstrated to exist for a wide variety of experimental conditions (Tables 2 and 3). The results imply that there is a fixed proportion of the catalytic power of PGH synthase utilized for each of the two substrates, arachidonic acid and ferulic acid.

The Tyrosyl Radical Mechanism. There are two mechanisms in the literature which describe the cycloxygenase and peroxidase reactions. The first is the tyrosyl radical mechanism described in eqs 5–11. The tyrosyl radical, detected by ESR spectroscopy (Karthein et al., 1988), is formed with a rate constant of 65 s^{-1} (Dietz et al., 1988) by the spontaneous conversion of the conventional Fe^{IV}=O porphyrin π-cation radical form of compound I to an Fe^{IV}=O tyrosyl radical possessing the same number of oxidizing equivalents. Eqs

5–9 are based on the *Perspectives in Biochemistry* article by Smith et al. (1992). We have added eqs 10 and 11 since it is necessary when discussing a total mechanism for a free radical chain mechanism to include chain-termination steps. Removal of both compounds I and II by competition of phenol with arachidonic acid interferes with the arachidonic acid reaction. Phenolic free radicals do not react with molecular oxygen, but typically react with each other (Hewson & Dunford, 1976), so they act as efficient chain terminators. Thus eqs 5–11 lead to a prediction that increasing amounts of the phenol ferulic acid would lead to an ever increasing ratio of ferulic acid to arachidonic acid reacted, which is clearly not the case. It is evident that neither an uncontrolled arachidonic acid reaction nor an arachidonic acid reaction at a rate which is faster than that of ferulic acid reaction can occur.

Hydrogen Atom Abstraction from Arachidonic Acid by Compound I Followed by Compound II Reduction by Ferulic Acid. The alternative to the tyrosyl radical hypothesis is that a conventional compound I is responsible for hydrogen atom abstraction, as shown by eqs 5, 12, 8, 13, and 11, in sequence, listed in the introduction. These equations predict that no PGG_2 would be detected in the stimulated reaction, in agreement with literature results obtained with ram seminal vesicle microsomes or purified enzyme (Nutgeren & Hazelhof, 1973; Smith & Lands, 1971; Egan et al., 1976; Graff, 1982). As explained in the original proposal of Hsuanyu and Dunford (1992), this reaction may consist of abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid by the Fe^{IV}=O group of compound I (a cytochrome P-450-like reaction), followed by proton transfer to a distal basic group of the enzyme and electron transfer to the porphyrin ring, as occurs in the normal peroxidatic mechanism of compound II formation (Dunford, 1991). Alternatively, as suggested by a referee, the porphyrin π-cation radical of compound I may play the role we have suggested for the Fe^{IV}=O group. We cannot distinguish between these two possiblities. Our present results confirm the prediction, from the sum of equations 5, 12, 8, 13 and 11, of a 2:1 ratio of ferulic acid reacted/arachidonic acid reacted, and this excludes the tyrosyl radical chain mechanism. The abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid by a conventional compound I implies that arachidonic acid binds and reacts preferentially with compound I and that ferulic acid reacts preferentially with compound II (Hsuanyu & Dunford, 1992).

PGH synthase reconstituted with manganese porphyrin exhibits considerable cyclooxygenase activity but little peroxidase activity (Lassmann et al., 1991; Odenwaller et al., 1992; Strieder et al., 1992). Manganese-substituted yeast cytochrome *c* peroxidase exhibits properties markedly different from those of the iron holoenzyme, with about 10% of the normal peroxidatic activity (Yonetani & Asakura, 1968). Manganese has preferred oxidation states different from those of iron. It is not surprising that the manganese-substituted PGH synthase displays reactivities different from those of the iron enzyme. Its mechanism of action is as yet unknown. PGH synthase mutants also display different ratios of cyclooxygenase to peroxidase activities. Tyr-385 is essential for the cyclooxygenase activity and His-309, His-207, and His-388 are essential for both PHG synthase activities. Their replacement causes notable alterations in the enzyme functions (Shimokawa et al. 1990; Shimokawa & Smith, 1991). The cause may be alteration of the binding site of arachidonic acid and of the heme environment.

The important conclusion which can be drawn from the present study is that in the presence of reducing substrate an arachidonic acid chain reaction is not possible. At present, the only plausible alternative is a nonchain mechanism in which compound I of PGH synthase, an Fe^{IV}=O porphyrin π-cation radical, is responsible for hydrogen atom abstraction from arachidonic acid.

General Discussion. The tyrosyl radical mechanism and the peroxidase mechanism, as they have been outlined in the literature and in the introduction to this paper, can be distinguished in several ways. The predominant or exclusive product of arachidonic acid oxidation in the presence of phenol is PGH_2 (Nutgeren & Hazelhof, 1973; Smith & Lands, 1971; Egan et al., 1976; Graff, 1982). This supports the classical peroxidase mechanism in which PGG_2 is consumed stoichiometrically. The measurements of the rates of PGG_2 and PGH_2 formation will be useful in distinguishing between the two mechanisms, but these compounds as well as arachidonic acid do not possess suitable chromophores for stopped-flow spectrophotometric measurements. Generally, there is no problem in determination of the amount of phenolic products formed during arachidonic acid oxygenation except that secondary reactions such as polymerization can occur. Ferulic acid forms a single dimer in its horseradish peroxidase reaction (Bassoli et al., 1988). In this work have shown that the same product is formed in its PGH synthase reaction. Product analysis studies which include measurements of the amounts of both arachidonate and phenolate products will be of use in studying the stoichiometry of the reaction, but these remain to be done. In this study we established the stoichiometry between the amounts of arachidonic acid and the phenol ferulic acid reacted by using the kinetic method of initial rate measurements under steady-state conditions, by which the effects of secondary reactions and enzyme inactivation are minimized. Our kinetic experiments showed that there is a constant 2:1 stoichiometry between the amounts of the phenol ferulic acid and arachidonic acid reacted. This result contradicts the tyrosyl radical mechanism as outlined in eqs 5–11.

There is an alternative possibility which deserves consideration. The tyrosyl residue is a phenol, and thus it appears reasonable that it may participate in some fashion, although not in an independent free radical chain reaction. The following pair of reactions are a possibility:

$$\text{compound II} + PGG_2^{\bullet} \rightarrow \text{Cpd-I'-Tyr}^{\bullet} + PGG_2 \quad (9)$$

$$\text{Cpd-I'-Tyr}^{\bullet} + \text{HOPh} \rightarrow \text{compound II} + \text{OPh}^{\bullet} \quad (21)$$

The sum of eqs 9 and 21 is eq 13,

$$\text{HOPh} + PGG_2^{\bullet} \rightarrow \text{OPh}^{\bullet} + PGG_2 \quad (13)$$

part of the peroxidase mechanism. It is not possible at this time to distinguish between the two-step and one-step processes; both could be in operation. Evidence which supports the two-step hypothesis is that tyrosyl radicals were not detected in the presence of phenol (Lassman et al., 1991).

In conclusion, our results for reaction mixtures of arachidonic acid and a phenol preclude an independent tyrosyl radical chain mechanism, but they do not preclude participation of the tyrosyl residue and the radical in a different fashion. A classical peroxidase cycle, in which arachidonic acid is a preferred compound I substrate and a phenol such as ferulic acid reduces compound II, can account for all of the results.

REFERENCES

Bakovic, M., & Dunford, H. B. (1993) *Biochemistry 32*, 833–840.

Bassoli, A., DiGregorio, G., Rindone, B., Tollari, S., Chioccara, F., & Salmona, M. (1988) *Gazz. Chim. Ital. 118*, 763–768.

Beers, R. J., & Sizer, I. W. (1952) *J. Biol. Chem. 195*, 133–140.

Burton, G., & Ingold, K. U. (1989) in *CRC Handbook of Free Radicals and Antioxidants in Biomedicine* (Miguel, J., Quintanilha, A. T., & Weber, H., Eds.) Vol. II, pp 29–43, CRC Press, Boca Raton, FL.

Dietz, R., Nastainczyk, W., & Ruf, H. H. (1988) *Eur. J. Biochem. 171*, 321–328.

Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 2, pp 1–24, CRC Press, Boca Raton, FL.

Egan, R. W., Paxton, J., & Kuehl, F. A., Jr. (1976) *J. Biol. Chem. 251*, 7329–7335.

Graff, G. (1982) in *Methods in Enzymology* (Lands, W. E. M., & Smith, W. L., Eds.) Vol. 86, pp 376–385, Academic Press, New York.

Hewson, W. D., & Dunford, H. B. (1976) *J. Biol. Chem. 251*, 6043–6052.

Hsuanyu, Y., & Dunford, H. B. (1992) *J. Biol. Chem. 267*, 17649–17657.

Karthein, R., Dietz, R., Nastainczyk, W. E., & Ruf, H. H. (1988) *Eur. J. Biochem. 171*, 313–320.

Kulmacz, R. J., & Lands, W. E. M. (1984) *J. Biol. Chem. 259*, 6358–6363.

Lambeir, A. M., Markey, C. M., Dunford, H. B., & Marnett, L. J. (1985) *J. Biol. Chem. 260*, 14894–14896.

Lassmann, G., Odenwaller, R., Curtis, J. F., DeGray, J. A., Mason, R. P., Marnett, L. J., & Eling, T. (1991) *J. Biol. Chem. 266*, 20045–20055.

MacDonald, I. D., & Dunford, H. B. (1989) *J. Inorg. Biochem. 37*, 35–44.

Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. R., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E., & Eling, T. E. (1984) *Mol. Pharmacol. 26*, 328–335.

Miyamoto, T., Ogino, N., Yamamoto, S., & Hayaishi, O. (1976) *J. Biol. Chem. 251*, 2629–2636.

Nugteren, D. H., & Hazelhof, E. (1973) *Biochim. Biophys. Acta 326*, 448–461.

Odenwaller, R., Maddipati, K. R., & Marnett, L. J. (1992) *J. Biol. Chem. 267*, 13863–13869.

Ohki, S., Ogino, N., Yamamoto, S., & Hayaishi, O. (1979) *J. Biol. Chem. 254*, 829–836.

Ohlsson, P. I., & Paul, K. G. (1976) *Acta Chem. Scand. B30*, 373–375.

Pagels, W. R., Sachs, R. J., Marnett, L. J., Dewitt, D. L., Day, J. S., & Smith, W. L. (1983) *J. Biol. Chem. 258*, 6517–6523.

Picot, D., & Garavito, R. M. (1989) in *Cytochrome P450: Biochemistry and Biophysics* (Schuster, I., Ed.) pp 29–36, Taylor and Francis, Philadelphia.

Robinson, J., & Cooper, J. M. (1970) *Anal. Biochem. 33*, 390–399.

Roth, G. J., Machuga, E. T., & Strittmatter, P. (1981) *J. Biol. Chem. 256*, 10018–10022.

Shimokawa, T., & Smith, W. L. (1991) *J. Biol. Chem. 266*, 6168–6173.

Shimokawa, T., Kulmacz, R. J., DeWitt, D. L., & Smith, W. L. (1990) *J. Biol. Chem. 265*, 20073–20076.

Smith, W. L., & Lands, W. E. M. (1971) *J. Biol. Chem. 246*, 6700–6702.

Smith, W. L., & Marnett, L. J. (1991) *Biochim. Biophys. Acta 1083*, 1–17.

Smith, W. L., Eling, E. T., Kulmacz, R. J., Marnett, J. L., & Tsai, A. L. (1992) *Biochemistry 31*, 3–7.

Strieder, S., Schaible, K., Scherer, H.-J., Dietz, R., & Ruf, H. H. (1992) *J. Biol. Chem. 267*, 13870–13878.

van der Oudera, F. J., Buytenhek, M., Nugteren, D. H., & van Dorp, D. A. (1977) *Biochim. Biophys. Acta 487*, 315–331.

Yonetani, T., & Asakura, T. (1968) *J. Biol. Chem. 243*, 3996–3998.