Kinetics of the Oxidation of *p*-Coumaric Acid by Prostaglandin H Synthase and Hydrogen Peroxide

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ABSTRACT: Steady-state kinetics of the oxidation of *p*-coumaric acid (CA) by prostaglandin H synthase and hydrogen peroxide was studied at 25 °C in 0.1 M phosphate buffer, pH 8.0, using a stopped-flow apparatus. The following evidence supports a mechanism in which CA serves as a reducing substrate for prostaglandin H synthase through two one-electron oxidation steps: (a) the oxidation product of CA is the same in the prostaglandin H synthase/hydrogen peroxide and the horseradish peroxidase/hydrogen peroxide systems; (b) an identical steady-state enzyme intermediate (compound II) is present in both systems; (c) CA stimulates the cyclooxygenase activity of prostaglandin H synthase; the concentration of CA that produces 50% stimulation, A_{50}, is 350 ± 30 μM. On the time scale of our experiments, the inactivation of prostaglandin H synthase by hydrogen peroxide was insignificant when CA was present. A molar absorptivity of 17.2 ± 0.9 mM^{-1} cm^{-1} at 300 nm was determined for CA which was used to follow the initial rate of disappearance of CA. The reaction of CA with hydrogen peroxide catalyzed by prostaglandin H synthase showed saturation behavior. An irreversible reaction mechanism for the steady-state kinetics of prostaglandin H synthase is proposed which is consistent with all of our experimental results. Under steady-state conditions, the second-order rate constants for the reactions of prostaglandin H synthase with hydrogen peroxide and prostaglandin H synthase–compound II with CA are (9.2 ± 0.1) × 10^5 and (2.5 ± 0.1) × 10^6 M^{-1} s^{-1}, respectively. Steady-state kinetic studies of prostaglandin H synthase can be performed by using stopped-flow spectrophotometry (dead time 1–5 ms) with considerable advantage over conventional spectrophotometry (dead time of the order of 1–5 s), in particular with regard to avoiding problems of enzyme inactivation.

p-Coumaric acid (CA)[1] is an important member of the hydroxycinnamate family which is widely distributed throughout the plant kingdom, and thus it is present in food and beverages derived from plants (Deshpande et al., 1984; Nergiz & Unal, 1991). Plant phenolases, laccases, and peroxidases are enzymes responsible for the oxidative metabolism of hydroxycinnamate compounds (Butt, 1977; Barz & Hoesel, 1977). Among them, cell wall bound peroxidases are generally regarded as the natural catalysts of oxidative polymerization of hydroxylated cinnamyl alcohols (Pang et al., 1989; Pedreño et al., 1987).

CA is a natural antioxidant (Dziedzic & Hudson, 1984), as are eugenol, cinnamic acid, and other natural aromatics (Simic, 1981). CA (Suzuki et al., 1990) and/or its synthetic analogs (Salnikova et al., 1989) are scavengers of the lipid peroxides and can protect against liver injury. Furthermore, CA is able to promote release of ferritin iron (Boyer et al., 1990) and to activate NADH (Pedrẽno et al., 1987) and indoleacetic acid peroxidatic metabolism (Shuto et al., 1986). CA isomers are the inhibitors of yeast phenylalanine ammonia-lyase (Hodgins, 1971) and the activators of electron transport and the inhibitors of photophosphorylation (Muzafarov et al., 1989), and CA and its amides and esters are tyrosine kinase inhibitors and anticancer agents (Goto et al., 1991). Among other naturally occurring inhibitors of chemical carcinogenesis, *p*- and *o*-hydroxycinnamic acids cause mild inhibition of β-propiolactone-induced neoplasia (Wattenberg, 1979). Plant peroxidase, HRP, serves as a mediator in oxidative coupling of hydroxycinnamate reactive intermediates with anilines (Berry & Boyd, 1985), polysaccharides (Whitmore, 1976; Markwalder et al., 1983), and amino acids and proteins (Van Sumere et al., 1973; Friend, 1977).

A wide range of compounds may be cooxidized by PGHS during prostaglandin biosynthesis including drugs, carcinogens, and natural products (Marnett et al., 1975; Plé & Marnett, 1989; Boyd et al., 1983; Sivarajah et al., 1982; Marnett & Eling, 1983; Ham et al., 1979). During catalysis PGHS acts first as a cyclooxygenase, oxidizing AA to PGG_2, and then as a peroxidase, reducing the hydroperoxide PGG_2 to PGH_2 (Hamberg et al., 1974). Structural and catalytic properties of PGHS have been reviewed recently (Marnett & Maddipati, 1991; Smith & Marnett, 1991; Smith et al., 1992).

PGHS peroxidase has a double role in catalysis: to produce PGH_2 and to protect the enzyme from oxidative damage by utilizing reducing substrates other than PGG_2 (Markey et al., 1987, Hsuanyu & Dunford, 1990a,b, 1992a,b). In the light of recent findings (Lassmann et al., 1991, Hsuanyu & Dunford, 1992a,b), it seems that the tyrosyl radical produced during the peroxidase reaction is not a catalytically competent intermediate in catalysis. It inactivates the enzyme in the absence of sufficient protective reducing substrate.

The present paper describes steady-state experiments on a time scale where enzyme inactivation by hydrogen peroxide does not significantly affect the reaction cycle. We propose a mechanism for the PGHS peroxidatic oxidation of CA that may be biologically important, and we make a comparison of the PGHS and HRP systems in their reactions with CA.

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[1] Abbreviations: CA, *p*-coumaric acid; HRP, horseradish peroxidase; PGHS, prostaglandin H synthase (EC 1.14.99.1); AA, arachidonic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; DDC, diethyldithiocarbamate; ε, molar absorptivity; BSA, bovine serum albumin; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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EXPERIMENTAL PROCEDURES

Materials. AA, CA, and DDC were purchased from Sigma. Tween 20 was purchased from J. T. Baker and DE-53 ion-exchange resin from Whatman. HRP (grade I) was obtained from Boehringer-Mannheim (A_{280nm}/A_{430nm} ratio of 3.1 after dialysis). The concentration of the HRP was determined at 403 nm using a molar absorptivity of 1.02×10^5 M^{-1} cm^{-1} (Ohlsson & Paul, 1976).

H_2O_2 (30%) was purchased from Fisher. The concentrations of H_2O_2 diluted stock solutions were determined by the peroxidase assay (Cotton & Dunford, 1973) and/or by the absorbance change at 240 nm using an ϵ of 43.6 M^{-1} cm^{-1} (Kulmacz, 1986).

PGHS of high purity was obtained from ram seminal vesicle microsomes using a published procedure as modified (Marnett et al., 1984; MacDonald & Dunford, 1989a). Unless otherwise specified, 82% holoenzyme having a specific activity of 69 μM O_2 (mg of protein)$^{-1}$ min^{-1} was used in the kinetic experiments. No hematin was added. Protein concentration was determined by the Bio Rad protein assay using BSA as a standard. The concentration of PGHS was determined at 410 nm using a molar absorptivity of 123 mM^{-1} cm^{-1} (Kulmacz & Lands, 1984).

PGHS Cyclooxygenase Reaction. The effect of CA on the PGHS cyclooxygenase activity was determined by monitoring oxygen uptake with a Yellow Springs Instrument Model 53 oxygen monitor. The incubation mixture without hematin contained 0.1 M phosphate buffer (pH 8.0), either 17, 18, or 20 nM enzyme, 200 μM AA, and CA in varying concentrations. The reaction rate was determined from the maximum slope of the oxygen consumption curve by assuming that 2 mol of oxygen was consumed per mole of AA. The oxygen concentration in 0.1 M phosphate buffer was calibrated to be 0.23 mM at 30 °C (Robinson & Cooper, 1970).

PGHS and HRP Peroxidase Reactions. Optical absorption measurements on a conventional time scale were made on a Cary 219 spectrophotometer. To determine if CA is a reducing substrate of PGHS peroxidase, separate assays with PGHS/H_2O_2 and HRP/H_2O_2 systems were performed by comparing spectral changes in the 227–387-nm region. The drop in CA absorbance at 286 nm was used as a parameter in setting optimal conditions for the kinetic steady-state experiments. There were no significant spectral interferences from H_2O_2 (up to 8 mM) and PGHS (up to 0.1 mM) with the CA spectra. However, there was some overlap in the far-UV region at the lowest CA concentration used (5 μM) so that the absorbance at 300 nm was chosen as more convenient for the kinetic steady-state measurements.

Determination of Molar Absorptivity of CA. The molar absorptivity of CA at 300 nm was determined at 25 ± 1 °C in 0.1 M phosphate buffer (pH 8.0). The CA was recrystallized from hot water and had a purity of 98% as determined by elemental microanalysis. Beer's law was obeyed over the range 5–71 μM CA. The molar absorptivity of CA calculated from four Beer's law plots was 17.2 ± 0.9 mM^{-1} cm^{-1}.

Steady-State Kinetic Measurements. The steady-state experiments were performed on a Photal (formerly Union Giken) RA601 stopped-flow apparatus. One compartment was used for the H_2O_2 solutions, the other for the PGHS and CA, all in 0.1 M phosphate buffer (pH 8.0). After 3 min of incubation at 25 ● 0.5 °C, the initial rate of CA disappearance was determined at 300 nm from the slope of the linear trace obtained after fast (2 ms) mixing of all the components. The initial rates were determined up to 1.5 s after the reaction was started. A stoichiometry of 2 mol of CA oxidized per mole of H_2O_2 reduced was used in calculations.

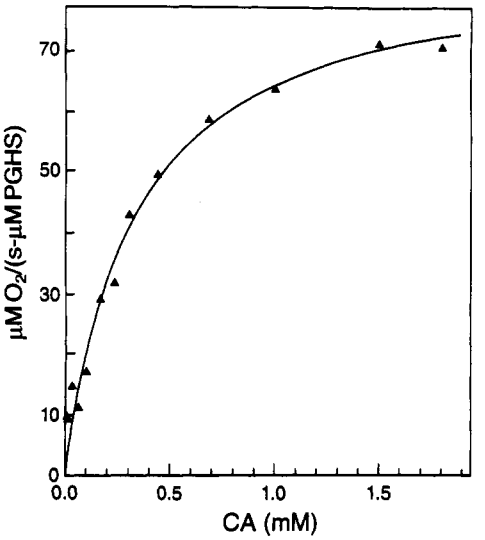

FIGURE 1: Stimulation by CA of oxygen uptake during PGHS-catalyzed prostaglandin biosynthesis. Plot of micromolar O_2 consumed per second per micromolar PGHS versus [CA] at 30 ± 1 °C in 0.1 M phosphate buffer (pH 8.0) with 200 μM AA and without hematin. Data points are the average of three determinations.

Curve Fittings and Calculations. A nonlinear regression data analysis program (ENZFITTER, Elsevier-Biosoft, Cambridge, UK) was used for curve fittings and calculations. The initial rate (v) data versus substrate (S) concentration were fitted to an equation of the form

$$v/[E]_{tot} = A[S]/(B + [S]) \quad (1)$$

to determine the values of the parameters A and B.

In the PGHS cyclooxygenase reaction, the variables v and [S] represent the rate of oxygen consumption and [CA], respectively. Parameter A is k_{cat} for cyclooxygenase activity; parameter B is A_{50}, i.e., the value of [CA] that produces half the maximum of the oxygen consumption.

In the PGHS peroxidase reaction, the variables v and [S] are the rate of H_2O_2 disappearance and [H_2O_2], respectively. Parameters A and B were fitted to the linear equations as functions of [CA].

RESULTS

Effect of CA on PGHS Cyclooxygenase Activity. CA stimulated the cyclooxygenase activity of PGHS as determined by measuring the oxygen consumption during the catalytic oxidation of AA. The stimulation of cyclooxygenase activity was dependent upon CA concentration and showed saturation behavior (Figure 1). The k_{cat} for the oxygen uptake in the presence of CA was 87 ± 3 s^{-1} when 200 μM AA was used. The concentration of CA necessary to produce 50% stimulation (A_{50}) was 350 ± 30 μM. Concentrations of CA up to 1.8 mM did not inhibit the oxygen uptake. Measurements above 1.8 mM CA were limited by the low solubility of CA in phosphate buffer.

CA Oxidation by HRP and PGHS. CA strongly absorbs in the UV region with a maximum at 286 nm and a shoulder at 294 nm at pH 8.0 (Figure 2A–D,a). CA is not oxidized spontaneously with H_2O_2, but in the presence of catalytic amounts of either HRP or PGHS it is oxidized very quickly, giving identical products (Figure 2A-b and D-b). The isosbestic points between CA and its product are at 254 and 324 nm in the HRP/H_2O_2 system and at 253 and 323 nm in the PGHS/H_2O_2 system, essentially the same within experimental error. The product has a broad maximum at 290 nm and a shoulder at 302 nm and does not react further under the given experimental conditions. The oxidation of CA was

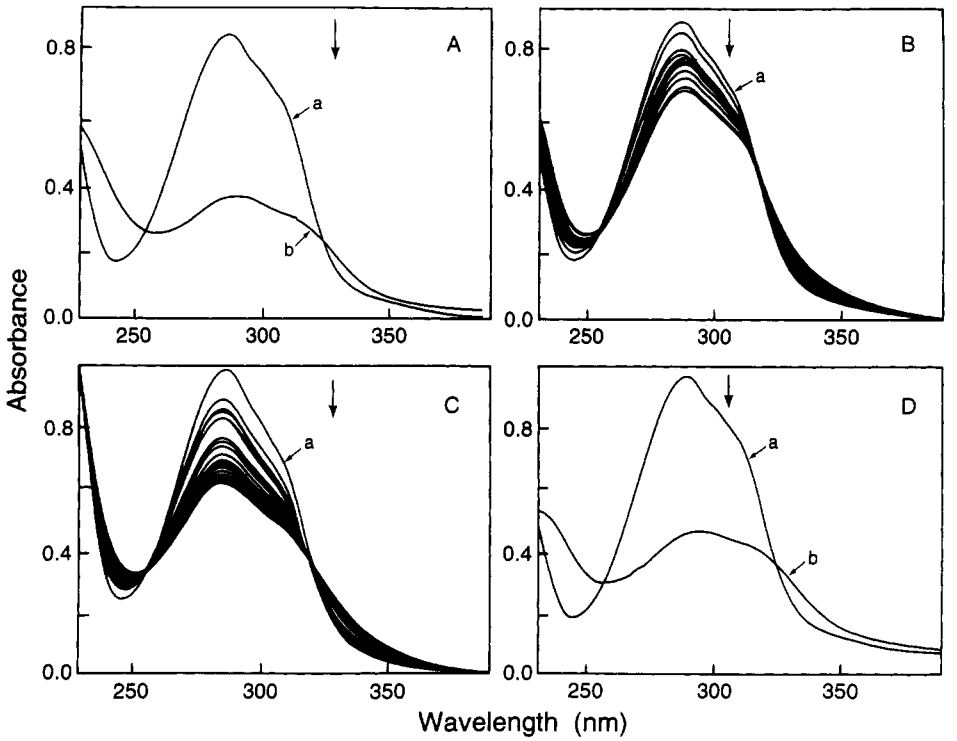

FIGURE 2: Oxidation of CA by HRP/H_2O_2 and PGHS/H_2O_2: (A) (a) Initial spectrum and (b) final spectrum (20 min) of CA (50 μM) after its reaction with 1 mM H_2O_2 and 25 nM HRP at 25 ● 0.5 °C in 0.1 M phosphate buffer (pH 8.0). (B) Repetitive scans (5 nm/s) taken over 5.5 min of CA (50 μM) during its reaction with 1 mM H_2O_2 and 25 nM PGHS. (C) Repetitive scans (5 nm/s) taken over 7 min of CA (50 μM) during its reaction with 0.25 mM H_2O_2 and 50 nM PGHS. (D) (a) Initial spectrum and (b) final spectrum (40 min) of CA under conditions as in (C).

complete at low concentration of H_2O_2 and high concentration of PGHS (Figure 2C,D), but it was inhibited at high concentration of H_2O_2 and low concentration of PGHS (Figure 2B).

Initial Rate Measurement for the Reaction of CA with PGHS/H_2O_2. Preliminary tests were made to find the relationship between rate, total enzyme concentration, and concentrations of both substrates. The rate of CA disappearance is a linear function of the enzyme concentration in the range of 20–50 nM enzyme at fixed concentrations of CA and H_2O_2. The intercept is not significantly different from zero and the slope is 41 ± 1 μM CA s^{-1} (nM PGHS)$^{-1}$. At concentrations above 50 nM, a saturation in PGHS peroxidase activity was observed. A linear dependence of the intial rates on CA concentration at fixed PGHS and H_2O_2 concentrations is also obtained with zero intercept and a slope of 2.1 ± 0.1 μM PGHS s^{-1}. A range in reducing substrate concentration of at least 1 order of magnitude was covered.

The assay period was short enough (1.5 s) to ensure that only a small fraction of the CA was utilized. Over the utilized concentration range of CA, 3–8% of the CA was reacted after 1.5 s. This was ascertained from the initial drop in absorbance at 286 nm by using rapid-scan measurements (data not shown).

The initial rate of CA disappearance was measured at 300 nm immediately after the steady-state conditions had been established: the concentration of the enzyme intermediate—PGHS–compound II—rapidly reaches its maximum value, which then remains constant for a time interval over which an initial steady-state rate of substrate disappearance can be measured. The change is absorbance was monitored at 426 nm (the isosbestic point between PGHS–compound I and PGHS–native enzyme) and 419 nm (the absorption maxima of PGHS–compound II) (Hsuanyu & Dunford, 1990a). The kinetic trace at the 426-nm isosbestic points (Figure 3) clearly indicates that PGHS–compound II is the dominant enzyme intermediate under steady-state conditions.

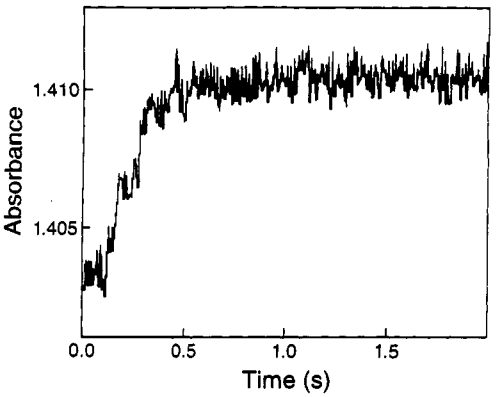

FIGURE 3: Kinetic trace of PGHS (0.3 μM) absorbance at 426 nm in the presence of 30 μM CA and 0.5 mM H_2O_2 in 0.1 M phosphate buffer (pH 8.0) at 25 ● 0.5 °C.

Initial rate data can be represented by an empirical linear equation of the form

$$[S] = \alpha + \beta t \quad (2)$$

[S] represents the concentration of the CA that has disappeared at time t. The coefficient α is the intercept; if negative, α could represent a lag period, but if positive, it could represent contamination by the product. As can be seen in Figure 4, both situations existed but they did not significantly affect the initial rate determination from slopes β over the 0.5–1.5-s time scale. Applying Beer's law, the initial rate of CA disappearance has been calculated from the change in absorbance as a function of time. Average values of β were determined from at least five measurements in which the relative error was usually less than 8%.

When the reaction period was extended to 5 s, a departure from linearity was observed (Figure 5). By increasing the H_2O_2 concentration the rate of the reaction was increased

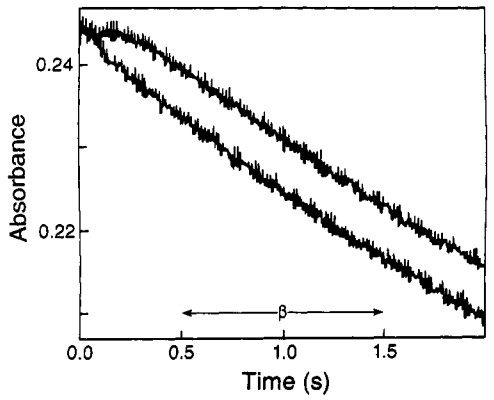

FIGURE 4: Repetitive kinetic traces of CA absorbance at 300 nm during its oxidation with PGHS and H_2O_2. The reaction mixture contained 20.35 μM CA, 0.382 mM H_2O_2, and 25 nM PGHS in 0.1 M phosphate buffer (pH 8.0) at 25 ± 0.5 °C; β is the coefficient from eq 2.

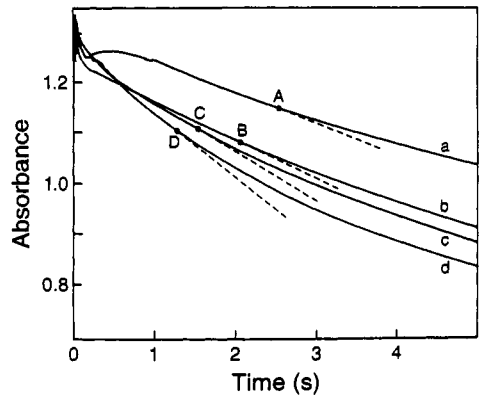

FIGURE 5: Hydrogen peroxide dependent oxidation of CA. The reaction mixture contained 69.85 μM CA, 51 nM PGHS, and (a) 0.271, (b) 0.596, (c) 1.953, or (d) 3.255 mM H_2O_2 in 0.1 M phosphate buffer (pH 8.0) at 25 ± 0.25 °C. Kinetic traces obtained at 300 nm. A–D: the points of departure from linearity.

and the departure from linearity was seen in even shorter times. The points of departure are labeled A–D in Figure 5.

Steady-State Kinetics for the Reaction of CA with PGHS/H_2O_2. The linear portions in the traces on a short time scale (Figure 4) allowed us to obtain the kinetic data presented in Figure 6. In the range of 5.4–55.1 μM CA, the initial rate was dependent upon both H_2O_2 and CA concentrations and showed saturation behavior. After fitting the experimental data to eq 1, parameters A and B were obtained (Table I).

Kinetic parameters obtained from steady-state analysis can be fitted to a rectangular hyperbola (eq 1) by using the operational equations (a) from the irreversible reaction mechanism which is widely accepted for HRP (George, 1952) or (b) in the manner of reversible Michaelis–Menten reaction kinetics (Kulmacz, 1986; Marshall & Kulmacz, 1988) that is incorrectly applied for HRP systems (Pedrēno, 1987; Seifullina et al., 1988).

Based on the results obtained in this study the most plausible mechanism for the oxidation of CA by H_2O_2 catalyzed by PGHS is the irreversible reaction mechanism (mechanism I) presented in Figure 7. The distinction between the mechanisms is based upon the ratio $2A/B$. In mechanism I, the irreversible mechanism, this ratio is equal to k_1 (Appendix, eq 12) and is independent of [CA]. For both mechanisms II and III (Figure 7, and Appendix eqs 20 and 26), the ratio of $2A/B$ is a hyperbolic function of [CA], which is contrary to the experimental results. A plot of $2A$ versus B (Table I, Figure 8) is linear with a slope of $k_1 = (9.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and an intercept not significantly different from zero.

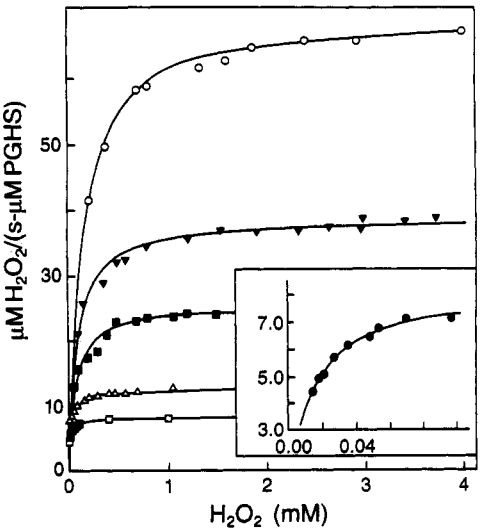

FIGURE 6: Steady-state initial rates at unit total PGHS concentration of the reduction of the H_2O_2 obtained at five concentrations of CA. CA concentrations are 5.44 (□), 10.18 (△), 20.35 (■), 30.04 (▼) and 55.1 (○) μM. PGHS concentrations are 25.5 nM for all experiments except those corresponding to 10.18 μM CA, where [PGHS] is 20.0 nM. Other conditions are given in Experimental Procedures. Inset: The initial part of the curve corresponding to 5.44 μM CA. The axes are the same as in the main part of the figure. All curves are theoretical curves obtained by fitting the data to eq 1. Data points are the experimental values.

Table I: Steady-State Parameters for the Peroxidase Reaction of PGHS with H_2O_2 and CA[a]

	parameter[b]	
CA (μM)	A (s^{-1})	B (μM)
5.44	8.1 (0.1)[c]	11.9 (0.1)
10.18	12.57 (0.02)	23.5 (0.1)
20.35	25.3 (0.2)	60.6 (0.1)
30.04	38.7 (0.3)	85.6 (0.1)
55.19	70.6 (0.5)	148.0 (0.1)

[a] Obtained from plotting initial rate of H_2O_2 disappearance against H_2O_2 concentrations at constant concentrations of CA. 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 eq 1. [c] Standard deviation.

Recovery of the native PGHS from PGHS–compound II is the rate-controlling step. Consequently, $k_2 \gg k_3$ and a simplified rate eq 3 is obtained:

$$\frac{-d[H_2O_2]/dt}{[E]_{tot}} = \frac{(1/2)k_3[CA][H_2O_2]}{(k_3/k_1)[CA] + [H_2O_2]} \quad (3)$$

The parameters A and B become

$$A = (1/2)k_3[CA] \quad (4)$$

$$B = (k_3/k_1)[CA] \quad (5)$$

Plots of parameters A and B as functions of [CA] thus yielded straight lines with slopes of $k_3/2$ (1.27 ± 0.04 μM^{-1} s^{-1}) and k_3/k_1 (2.80 ± 0.08), respectively. As expected from the forms of eqs 4 and 5, the intercepts were not significantly different from zero. The rate constants k_1 (9 ± 1) × 10^5 M^{-1} s^{-1} and $k_3 = (2.5 \pm 0.1) \times 10^6$ M^{-1} s^{-1} then were obtained. The values of k_1 determined directly from mechanism I (Appendix, eq 12) or by using the simplified version (eqs 4 and 5) are in a good agreement.

DISCUSSION

Our kinetic and spectroscopic data demonstrate that the naturally occurring hydroxycinnamate derivative, CA, func-

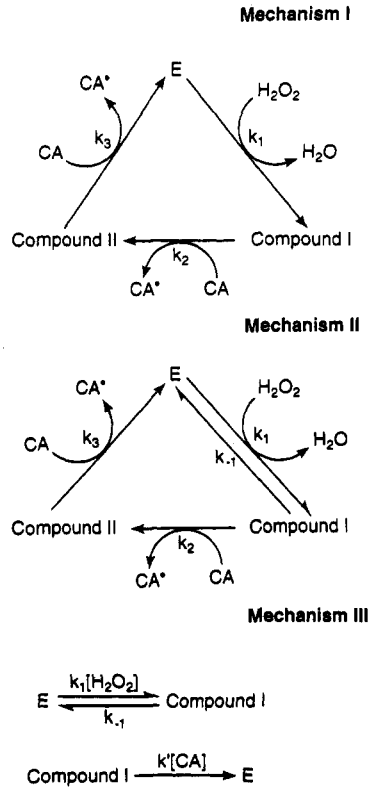

FIGURE 7: Three possible mechanisms for the oxidation of CA by H_2O_2, catalyzed by PGHS. Symbols for mechanism I: E, native enzyme; k_1, rate constant for compound I formation; k_2, rate constant for one-electron reduction of compound I by CA; k_3, rate constant for one-electron reduction of compound II by CA. Symbols for mechanisms II and III same as for mechanism I with the following additions: k_{-1}, rate constant for compound I back to the native enzyme (mechanisms II and III); k', rate constant for two-electron reduction of compound I by CA (mechanism III).

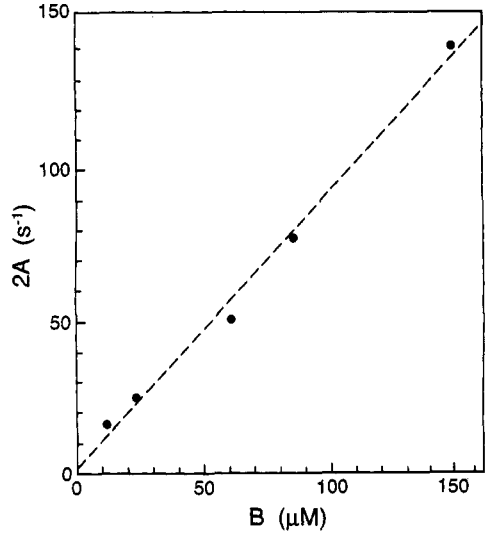

FIGURE 8: Linear dependence of parameter $2A$ versus parameter B for peroxidase activity, showing that mechanism I is correct (Appendix, eq 12). The numerical values are from Table I. The slope of the line is $(9.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1}.

tions as a reducing substrate of PGHS peroxidase and stimulates PGHS cyclooxygenase activity.

On longer time scales the inactivation of PGHS, particularly in the presence of higher concentrations of hydroperoxides, has a significant effect on a normal catalytic cycle so that either product yields were underestimated (Ohki et al., 1987; Markey et al., 1987) or kinetic parameters were corrected for the inactivation (Kulmacz, 1986). On the other hand, rapid-scan spectral and transient-state kinetic studies (Hsuanyu & Dunford, 1990a,b, 1992a,b) showed that reducing substrates are capable of protecting the enzyme from oxidative inactivation. This prompted us to believe that the difficulties that usually appear in steady-state kinetic studies due to rapid PGHS inactivation can be overcome by using stopped-flow spectrophotometry. In the PGHS peroxidase reaction with H_2O_2 as a oxidizing substrate and CA as a reducing substrate, we were able to obtain accurate kinetic data without inhibition on a time scale up to 1.5 s after fast mixing all of the components. H_2O_2 was chosen as an oxidizing substrate instead of organic hydroperoxides because it does not inactivate PGHS as much as the organic hydroperoxide 15-HPETE. The inactivation constants are 30 M^{-1} s^{-1} for H_2O_2 versus 2×10^4 M^{-1} s^{-1} for 15-HPETE (Marshall & Kulmacz, 1988).

We propose the irreversible reaction mechanism for the PGHS peroxidase reaction (mechanism I) for the first time for steady-state conditions. The results obtained earlier in our laboratory under transient-state conditions (Lambeir et al., 1985; MacDonald & Dunford, 1989b; Hsuanyu & Dunford, 1990a,b, 1992a,b) were all interpreted in terms of mechanism I.

Mechanism I is a three-step cycle involving a two-electron enzyme oxidation (native enzyme → compound I) followed by two one-electron reductions (compound I → compound II followed by compound II → native enzyme). Our rate constant determined for the first step (in detail, native enzyme + H_2O_2 → compound I + H_2O) is $(9.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1}. This is almost 2 orders of magnitude higher than what Dietz et al. (1988) estimated from a kinetic simulation of the reaction mechanism with tyrosyl radical formation as one of the steps in the PGHS cycle (1×10^4 M^{-1} s^{-1} without a reducing substrate). It is 1 order of magnitude greater than the value of 9.1×10^4 M^{-1} s^{-1} obtained by Kulmacz (1986) in a study using H_2O_2 and TMPD as the oxidizing and reducing substrates. In the latter study, the normal peroxidase cycle with three irreversible steps was approximated by mechanism III, containing one reversible and one irreversible step. An apparent Michaelis constant K_M^{app} was introduced and the reaction was classified as obeying an ordered mechanism. Peroxidase kinetics obey a modified ping-pong mechanism and Michaelis constants, in the strict sense, are meaningless for irreversible reactions (Dunford, 1991). The different results obtained by Kulmacz (1986) can partly be attributed to the different mechanisms and partly to the different time scales; inactivation was a factor on the longer time scale.

Our results also show that the oxidation product of CA is identical with the product obtained in the HRP/H_2O_2 system, for which the irreversible reaction mechanism involving two enzyme intermediates is well established (Dunford & Stillman, 1976). Our rate constant for the reaction of PGHS–compound II with CA [$k_3 = (2.5 \pm 0.1) \times 10^6$ M^{-1} s^{-1}] is comparable with the rate constant for the reaction HRP–compound II with CA (5×10^6 M^{-1} s^{-1}) (Hodgson & Jones, 1989).

It is also clear that the inactivation of PGHS is irrelevant on a time scale up to 1.5 s and up to 5 mM H_2O_2. Under these conditions CA, is able to protect the enzyme from oxidative damage at concentrations as low as 5 μM (Figure 6). A recent investigation showed that phenol can completely prevent doublet formation and partially prevent formation of the singlet tyrosyl radical signal without inhibiting cyclooxygenase activity (Lassman et al., 1991). Hsuanyu and Dunford (1992a) also suggested that the tyrosyl radical is not a significant intermediate when any of the reducing substrates phenol, hydroquinone, or DDC are present. Our results are consistent with these findings and with the view that hydroperoxide-

dependent generation of the tyrosyl radical represents an inactivation step rather than the initiation step in the cyclooxygenase reaction.

The ability of CA to serve as a reducing substrate for PGHS peroxidase correlates well with its ability to stimulate PGHS cyclooxygenase activity. CA prevents autoinactivation of the PGHS probably not only by minimizing the level of unproductive higher oxidation states of PGHS but also by reducing the peroxyl radical of PGG_2, as Harvison et al. (1988) proposed for acetaminophen and Hsuanyu and Dunford (1992b) proposed for substituted phenols. Also, it was known that CA can scavenge lipid hydroperoxides (Suzuki et al., 1990; Salnikova et al., 1989). The rate constant of the overall reaction from AA to the formation of PGG_2 was estimated to be 4×10^4 M^{-1} s^{-1} at 25 °C (Hsuanyu & Dunford, 1992b), which is 2 orders of magnitude slower than the conversion of PGHS–compound II by CA. If carbon-centered radical formation (Schreiber et al., 1986) is the rate-controlling step in the cyclooxygenase reaction, then AA is unable to compete with CA for the enzyme intermediate compound I. It is reasonable to expect that CA reacts with PGHS–compound I in a manner similar to HRP–compound I, for which the rate constant is 1.2×10^7 M^{-1} s^{-1} (Hodgson & Jones, 1989).

Yet another aspect of the observed stimulation of AA metabolism by CA needs to be considered. Hsuanyu and Dunford (1992b) established a quantitative relationship between both stimulatory and inhibitory abilities of phenolic compounds for the PGHS cyclooxygenase and electronic and hydrophobic properties of phenolic ring substituents. The action of phenols is a complex combination of structural and electronic effects resulting in a competition of phenols with the AA not only for the enzyme higher oxidation states but also for the AA hydrophobic binding site. The hydrophobic effect strongly inhibits PGHS cyclooxygenase activity. Consequently, compounds such as *p*-hydroxybenzoic acid did not exhibit inhibition partly due to the presence of a very strong hydrophilic carboxyl group. In the case of CA, the stimulatory effect was also observed (Figure 1). At pH 8, the carboxyl group of CA is deprotonated and the hydroxyl group partially deprotonated (pK_1 and pK_2 are 4.64 and 9.45, respectively) (Sarjeant & Dempsey, 1979), which increases the hydrophilicity of CA and diminishes its competition for the AA binding site.

This study shows that PGHS can play a role in biologically important events such as oxidation of naturally occurring CA and related compounds. These observations are consistent with one-electron oxidations by PGHS compounds I and II to form phenoxyl radicals similarly to other phenols (Hsuanyu & Dunford, 1992b). The isosbestic point obtained in the ultraviolet CA spectrum (Figure 2B) indicates that a single product is formed during its reaction with PGHS and H_2O_2. At least three ways of dimerization of the CA radicals are possible: (a) through a 3,3′ linkage, as in case of eugenol (Basoli et al., 1988), ferulic acid (Markwalder & Neukom, 1976), phenol (Danner et al., 1973), and tyrosine (Bayse et al., 1972) oxidation with plant peroxidases; (b) through a β,β' linkage, as in the case of ferulic and caffeic acid oxidation with a peroxidase specific for this reaction from *Bulpeurum salicifolium* (Frías et al., 1991) and (c) through β-O-4 linkage, where similar products were formed when methylpherulate and isoeugenol were dimerized in the HRP/H_2O_2 system (Basoli et al., 1988).

Unlike ferulic acid oxidation with HRP/H_2O_2, in a PGHS-catalyzed reaction the ferulic acid undergoes O-demethylation to give caffeic acid similarly to the cytochrome P-450 catalyzed reaction (Basoli et al., 1988). Eugenol inhibits PGHS activity (Dewhirst, 1980) and chemiluminescence (Fotos et al., 1987). During the HRP-catalyzed oxidation of eugenol a transient quinone methide metabolite is formed (Thompson et al., 1989). Hodgson & Jones (1989) have found that CA enhances luminescence in the HRP/luminol/H_2O_2 system. Our data showed that CA stimulates PGHS activity, and it seems that cytotoxic quinone methide (a yellow intermediate with absorption maxima at 346 nm) is not the reaction intermediate in peroxidative oxidation of the CA by PGHS and HRP.

In conclusion, some important points concerning the mechanism of PGHS peroxidase have been established by using the stopped-flow method for steady-state kinetic measurements. An irreversible reaction mechanism similar to that for HRP is proposed. With CA and H_2O_2 as cosubstrates, we were able to obtain an accurate turnover number and rate constant for the rate-controlling step, which is the conversion of PGHS–compound II to the native enzyme, and for the PGHS–compound I formation. In view of the limitations imposed by the steady-state method, it is not possible to draw any conclusions about the relative magnitude of the rate of PGHS–compound I conversion to PGHS–compound II. However, the overall peroxidatic activity of PGHS for a substrate such as CA was defined and compared to the HRP system. In the case of CA, the reaction product is the same as that obtained in the HRP-catalyzed reaction and the reaction rates are comparable. Further studies are needed to yield more details about the PGHS peroxidase reaction mechanism. Even though PGHS and HRP can utilize the same reducing substrate, the products and intermediates can be dramatically different, as in case of ferulic acid (Basoli et al., 1988; Frías et al., 1991) or eugenol (Basoli et al., 1988; Thompson et al., 1989).

APPENDIX

Mechanisms are defined in Figure 7, steady-state conditions.

Mechanism I

rate:

$$\frac{d[\text{compound I}]}{dt} = k_1[E][H_2O_2] - k_2[\text{compound I}][CA] = 0 \quad (6)$$

$$\frac{d[\text{compound II}]}{dt} = k_2[\text{compound I}][CA] - k_3[\text{compound II}][CA] = 0 \quad (7)$$

mass balance:

$$[E]_{tot} = [\text{compound I}] + [\text{compound II}] + [E] \quad (8)$$

$$[E]_{tot} = \left(\frac{k_3}{k_2} + \frac{k_3[CA]}{k_1[H_2O_2]} + 1\right)[\text{compound II}] \quad (9)$$

initial rate expression:

$$\frac{-d[H_2O_2]}{dt} = \frac{-1}{2}\frac{d[CA]}{dt} = \frac{1}{2}k_3[CA][\text{compound II}] = \frac{1}{2}\frac{k_1k_2k_3[CA][H_2O_2][E]_{tot}}{k_2k_3[CA] + k_1(k_2 + k_3)[H_2O_2]} \quad (10)$$

Rearranging eq 10

$$\frac{\frac{-d[H_2O_2]}{dt}}{[E]_{tot}} = \frac{\frac{1}{2}\frac{k_2k_3}{(k_2+k_3)}[CA][H_2O_2]}{\frac{k_2k_3}{k_1(k_2+k_3)}[CA] + [H_2O_2]} = \frac{A[H_2O_2]}{B + [H_2O_2]} \quad (11)$$

$$A = \frac{1}{2}\frac{k_2k_3[CA]}{(k_2+k_3)} \quad B = \frac{k_2k_3[CA]}{k_1(k_2+k_3)} \quad \frac{2A}{B} = k_1 \quad (12)$$

The ratio $2A/B$ is constant independent of the CA concentration.

Mechanism II

rate:

$$\frac{d[\text{compound I}]}{dt} = k_1[E][H_2O_2] - (k_{-1} + k_2[CA])[\text{compound I}] = 0 \quad (13)$$

$$\frac{d[\text{compound II}]}{dt} = k_2[CA][\text{compound I}] - k_3[CA][\text{compound II}] = 0 \quad (14)$$

mass balance:

$$[E]_{tot} = [E] + [\text{compound I}] + [\text{compound II}] \quad (15)$$

$$[E]_{tot} = \left\{\frac{k_3(k_{-1} + k_2[CA])}{k_1k_2[H_2O_2]} + \frac{k_3}{k_2} + 1\right\}[\text{compound II}] \quad (16)$$

initial rate expression:

$$\frac{-d[H_2O_2]}{dt} = \frac{-1}{2}\frac{d[CA]}{dt} = \frac{1}{2}k_3[CA][\text{compound II}] = \frac{1}{2}\frac{k_1k_2k_3[CA][H_2O_2][E]_{tot}}{k_3(k_{-1} + k_2[CA]) + k_1(k_3 + k_2)[H_2O_2]} \quad (17)$$

Rearranging eq 17

$$\frac{-d[H_2O_2]}{dt} = \frac{\frac{1}{2}\frac{k_2k_3}{(k_2+k_3)}[CA][H_2O_2]}{\frac{k_3(k_{-1} + k_2[CA])}{k_1(k_2+k_3)} + [H_2O_2]} = \frac{A[H_2O_2]}{B + [H_2O_2]} \quad (18)$$

$$A = \frac{1}{2}\frac{k_2k_3[CA]}{(k_2+k_3)} \quad B = \frac{k_3(k_1 + k_2[CA])}{k_1(k_2+k_3)} \quad (19)$$

$$\frac{2A}{B} = \frac{k_1[CA]}{k_{-1}/k_2 + [CA]} \quad (20)$$

The ratio $2A/B$ is a hyperbolic function of the CA concentration.

Mechanism III

rate:

$$\frac{d[\text{compound I}]}{dt} = k_1[H_2O_2][E] - (k_1 + k'[CA])[\text{compound I}] = 0 \quad (21)$$

mass balance:

$$[E]_{tot} = [E] + [\text{compound I}] \quad (22)$$

$$[E]_{tot} = \{(k_{-1} + k'[CA])/k_1[H_2O_2] + 1\}[\text{compound I}] \quad (23)$$

initial rate expression:

$$\frac{-d[H_2O_2]}{dt} = \frac{-d[CA]}{dt} = k'[CA][\text{compound I}] = \frac{k_1k'[CA][H_2O_2][E]_{tot}}{(k_{-1} + k'[CA]) + k_1[H_2O_2]} \quad (24)$$

Rearranging eq 24

$$\frac{-d[H_2O_2]/dt}{[E]_{tot}} = \frac{k'[CA][H_2O_2]}{(k_{-1} + k'[CA])/k_1 + [H_2O_2]} = \frac{A[H_2O_2]}{B + [H_2O_2]} \quad (25)$$

$$A = k'[CA] \quad B = \frac{k_{-1} + k'[CA]}{k_1} \quad \frac{2A}{B} = \frac{2k_1[CA]}{k_{-1}/k' + [CA]} \quad (26)$$

The ratio $2A/B$ is a hyperbolic function of the CA concentration.

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