The inactivation and Catalytic Pathways of Horseradish Peroxidase with m-Chloroperoxybenzoic Acid

A SPECTROPHOTOMETRIC AND TRANSIENT KINETIC STUDY*

Jose Neptuno Rodriguez-Lopez‡‡, Josefa Hernández-Ruiz®, Francisco García-Cánovas**, Roger N. F. Thorneley‡ ‡, Manuel Acosta†, and Marino B. Arnao‡‡‡

From the §Nitrogen Fixation Laboratory, John Innes Centre, NR4 7UH Norwich, United Kingdom and the ¶Departamento de Biología Vegetal (Fisiología Vegetal) and **Departamento de Bioquímica y Biología Molecular A, Universidad de Murcia, 30100 Murcia, Spain

The kinetics of the catalytic cycle and irreversible inactivation of horseradish peroxidase C (HRP-C) reacting with m-chloroperoxybenzoic acid (mCPBA) have been studied by conventional and stopped-flow spectroscopy. mCPBA oxidized HRP-C to compound I with a second order rate constant $k_1 = 3.6 \times 10^7$ M$^{-1}$ s$^{-1}$ at pH 7.0, 25 °C. Excess mCPBA subsequently acted as a one-electron reducing substrate, converting compound I to compound II and compound II to resting, ferric enzyme. In both of these reactions, spectrally distinct, transient forms of the enzyme were observed ($\lambda_{max} = 411$ nm, $\epsilon = 45$ mm$^{-1}$ cm$^{-1}$ for compound I with mCPBA, and $\lambda_{max} = 408$ nm, $\epsilon = 77$ mm$^{-1}$ cm$^{-1}$ for compound II with mCPBA). The compound I-mCPBA intermediate (shown by near infrared spectroscopy to be identical to P965) decayed either to compound II in a catalytic cycle ($k_2 = 6.4 \times 10^4$ s$^{-1}$) or, in a competing inactivation reaction, to verdohemoprotein ($k_3 = 3.3 \times 10^3$ s$^{-1}$). Thus, a partition ratio of $r = 2$ is obtained for the inactivation of ferric HRP-C by mCPBA. The intermediate formed from compound II with mCPBA is not part of the inactivation pathway and only decays via the catalytic cycle to give resting, ferric enzyme ($k_4 = 1.0 \times 10^3$ s$^{-1}$). The results are compared with those from earlier steady-state kinetic studies and demonstrate the importance of single turnover reactions. The results are discussed in terms of the physiologically relevant reactions of plant peroxidases with hydrogen peroxide.

Horseradish peroxidase (HRP)1 (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) is an extracellular plant enzyme involved in the formation of free radical intermediates for the polymerization and cross-linking of cell wall components, for the oxidation of secondary metabolites essential for certain pathogenic defense reactions, and for the regulation of cell growth and differentiation (1). There are more than 30 isoforms of HRP, which are usually classified, according to isoelectric point, into three major groups: acidic, neutral, and basic (2). The slightly basic (cationic) horseradish peroxidase C (HRP-C) is the most studied isoform, since it constitutes approximately 50% of the peroxidase content of horseradish root and is commercially available because of its use in clinical analysis and biotransformations (see Refs. 3–5 for reviews of its structure and function).

HRP-C is a monomeric ($M_r$ of 33,922), glycosylated (18% by mass) enzyme that contains a single high spin ferric protoporphyrin IX prosthetic group and two calcium ions (6–8) that are necessary for competent folding of the recombinant enzyme after expression in Escherichia coli (9). The catalytic cycle is initiated by a rapid ($k_1 > 10^7$ M$^{-1}$ s$^{-1}$) 2e$^-$ oxidation of the enzyme by hydrogen peroxide (or other organic peroxides) to give a green enzyme intermediate, compound I, with the heme iron oxidized to the oxyferryl state (Fe(IV)=O) and a cation radical on the porphyrin ring. Compound I formation involves at least two reactions; formation of an intermediate enzyme-hydroperoxide complex (10), followed by heterolytic cleavage of the oxygen-oxygen bond. Histidine 42 and arginine 38, located in the distal heme cavity, are key residues that modulate both these reactions (11, 12). In order to complete a peroxidation cycle, compound I is reduced back to the resting Fe(III) state by two successive single electron transfer reactions from reducing substrate molecules, the first yielding a second enzyme intermediate, compound II. Both of these reactions can yield free radical products that can undergo subsequent chemistry. The rate of the peroxidation cycle usually depends on the nature of the reducing substrate, with the reduction of compound II to resting enzyme being rate-limiting (3).

In the absence of reducing substrates, excess hydrogen peroxide can react with compound I as an electron donor (reductant) in a catalase-like two-electron process that results in the formation of molecular oxygen or in two single-electron transfers in which compound II, compound III (Fe(II)=O), and superoxide radical anion ($O_2^-)$ are formed (13–15). Importantly in the context of this paper, a competing enzyme inactivation takes place that results in the generation of a number of inactive chromophores with the final product being a verdohemoprotein, P670 (16–19). A global reaction scheme that includes all the previously identified partial reactions of the catalytic cycle and the inactivation pathway has been developed using experimentally determined steady-state kinetic parameters for the HRP-C/H$_2$O$_2$ system (20). It was concluded that the cata-

---

* Received for publication, July 30, 1996, and in revised form, October 29, 1996

†‡‡ To whom correspondence should be addressed: Departamento de Biología Vegetal (Fisiología Vegetal), Facultad de Biología, Campus Espinardo, Universidad de Murcia, 30100 Murcia, Spain. Tel.: 34-68-307100; Fax: 34-68-363963; E-mail: marino@fcu.um.es.

§ Senior research scientist of the EU Human Capital and Mobility Program Network “Peroxidases in Agriculture and the Environment.” (Contract ERB CHRX-CT92-00120) and from Comision Interministerial de Ciencia y Tecnologia (Spain) Project ALI95-0487. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡‡ To whom correspondence should be addressed: Departamento de Biología Vegetal (Fisiología Vegetal), Facultad de Biología, Campus Espinardo, Universidad de Murcia, 30100 Murcia, Spain. Tel.: 34-68-307100; Fax: 34-68-363963; E-mail: marino@fcu.um.es.

1 The abbreviations used are: HRP, horseradish peroxidase; HRP-C, horseradish peroxidase isozyme C; mCPBA, m-chloroperoxybenzoic acid; ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid).
lase-like and compound III generating activities of HRP-C protect against inactivation and that the extent of inactivation is determined by the reactivity of a common intermediate, the complex formed between compound I and a second equivalent of H$_2$O$_2$ (18, 20). Consequently, with excess H$_2$O$_2$ as the only reducing substrate, the enzyme is completely inactivated after only ~600 turnovers (20). A simplified form of the reaction scheme has recently been developed to enable a more rapid, comparative analysis of data for isoenzymes from diverse sources (21) and to determine the effects of selected mutations in recombinant HRP-C (22).

A complementary approach to understanding the inactivation mechanism of HRP-C utilizes the competent, xenobiotic substrate, meta-chloroperoxybenzoic acid (mCPBA) instead of hydrogen peroxide. Compound I formation results in the release of the parent carboxylic acid instead of water (23) (Scheme I). Importantly, HRP-C in the ferric, compound I, and compound II states has a high affinity for mCPBA possibly because of its structural resemblance to reducing organic substrates such as benzohydroxamic acid (24–26) and indole-3-acetic acid (27, 28). These affinities permit single turnover experiments, with an excess of enzyme over substrate, using rapid-scan stopped-flow spectrophotometry. These conditions allow a precise determination of the values of the rate constants for the partial reactions shown in Table I in the absence of subsequent reactions that would occur at significant rates with an excess of mCPBA. In addition, the absence of the catalase-like cycle (13, 26, 29) causes the average number of turnovers effected by each molecule of enzyme before inactivation (r) to drop to very low values, typically 2 or 3 (22, 26, 30). This simplifies the kinetic analysis and aids the identification of intermediates in both the catalytic cycle and on the inactivation pathway, facilitates simulations of the complex absorbance changes, and allows a more rigorous test of our proposed mechanism of inactivation shown in Scheme I than was possible using pseudo-steady-state kinetics. In addition it has allowed us to show unambiguously that the reactivity of the complex formed between compound I and mCPBA is the key intermediate that determines the extent of inactivation. The results with mCPBA validate our earlier conclusions for the reactions of HRP-C with hydrogen peroxide, where it was only possible to use pseudo-steady-state kinetics, and allow us to discuss with greater confidence the physiological implications.

**MATERIALS AND METHODS**

**Reagents**—Horseradish peroxidase isoenzyme C was purchased from Biozyme (Type HRP-4B) and used without further purification. Characterization by electrophoresis and isoelectric focusing (21) revealed a band for peroxidase activity at pH 8.5, typical of a basic isoenzyme. Its concentration was determined spectrophotometrically using ε$_{403 nm}$ = 102 mm$^{-1}$ cm$^{-1}$. A specific activity of 1.322 mmol mg$^{-1}$ min$^{-1}$ was determined using 0.2 mm H$_2$O$_2$ and 0.5 mm ABTS as the reducing substrate in 50 mm glycine-HCl buffer, pH 4.5 at 25 °C. The preparations used for this study had ratios of absorbance at 403/280 nm (RZ value) of 3.2. mCPBA in the solid state (Aldrich) containing m-chlorobenzoic acid as an impurity was recrystallized from light petroleum ether (boiling point 40–60 °C)/diethyl ether (3:1, v/v) as described by Davies et al. (24), to give material that was more than 96% pure (NMR analysis and single melting point at 92–94 °C). Solutions of mCPBA were made up in 1:1 (v/v) absolute ethanol/water immediately prior to use. The concentration was determined using ε$_{423 nm}$ = 8,940 mmol$^{-1}$ cm$^{-1}$ allowing for the percentage purity obtained by NMR (26). Reagent grade H$_2$O$_2$ (30% v/v) was obtained from BDH, and its concentration determined spectrophotometrically using ε$_{400 nm}$ of 43.6 mm$^{-1}$ cm$^{-1}$ (31). The ABTS was supplied by Sigma as a diammonium salt, and solution concentrations were determined using ε$_{400}$ of 36 mm$^{-1}$ cm$^{-1}$ (32).

**Equipment**—Transient kinetics were monitored with a stopped-flow spectrophotometer (model SF-51), Hi-Tech Scientific, Salisbury, United Kingdom. Data were recorded through an RS232 interface with a microcomputer. Stopped-flow rapid-scan spectrophotometry was carried out with the same sample handling unit equipped with an MG-6000 diode array system. Spectrophotometric measurements were made with a Perkin-Elmer Lambda-2 UV-visible spectrophotometer controlled by an Olivetti PCS/386SX computer running dedicated software. The temperature was controlled at 25 or 10 °C using a Techne C-400 circulating bath with an integral heater-cooler system.

**Pre-steady-state Kinetics**—Compound I formation was monitored at 395 nm (isosbestic for compounds I and II). Compound II formation from compound I was monitored at 412 nm (isosbestic for compound II and the resting enzyme). Compound I was generated by mixing enzyme with an equimolecular amount of H$_2$O$_2$ in a simple pre-mixing device attached to one of the transport syringe ports on the stopped-flow apparatus and transferred directly to a drive syringe for use within 5 min of preparation. Compound II was made from compound I (prepared as described above) by oxidative titration with ascorbic acid. The disappearance of compound II was monitored at 424 nm (the isosbestic for compound I and the ferric enzyme). The formation of verdohemoprotein was followed at 670 nm. The determination of the reaction rates were carried out under single turnover conditions with [E]$_0$ ≫ [I]$_0$ (33). Pre-steady-state kinetic experiments were performed in 10 mm sodium phosphate buffer, pH 7.0, and the data analyzed by fitting the absorbance-time curve to exponential functions using a least-squares minimization procedure.

**Spectrophotometry**—The detection of enzyme intermediates and final products from reactions of ferric enzyme, compound I or compound II were carried out by mixing these species with a stoichiometric amount of mCPBA. Transient and final spectra were deconvoluted using a SPECFIT Global Analysis computer program (Spectrum Software Associates, Chapel Hill, NC). All experiments were in 10 mm sodium phosphate buffer, pH 7.0.

**Kinetic Simulations**—The kinetics of the reaction mechanism described in Scheme I is defined by a set of differential equations, whose numerical integration was carried out using a RSDM computer program provided by Dr. N. Millar. The differential equations are shown in Equations 1–6 below.

\[
[E] = k_1 [\text{compound II-mCPBA}] - k_2 [E][\text{mCPBA}] \quad (\text{Eq. 1})
\]

\[
[\text{compound I}] = k_3 [E][\text{mCPBA}] + k_4 \cdot [\text{compound I-mCPBA}] - k_5 \cdot [\text{compound I}] \quad (\text{Eq. 2})
\]

\[
[\text{compound I-mCPBA}] = k_6 [\text{compound I}][\text{mCPBA}] - (k_7 + k_8 + k_9) \cdot [\text{compound I-mCPBA}] \quad (\text{Eq. 3})
\]

\[
[E] = k_1 [\text{compound I-mCPBA}] \quad (\text{Eq. 4})
\]

\[
[\text{compound II}] = k_3 [\text{compound I-mCPBA}] + k_4 \cdot [\text{compound II-mCPBA}] - k_5 \cdot [\text{compound II}] \quad (\text{Eq. 5})
\]

\[
[\text{compound II-mCPBA}] = k_6 [\text{compound II}][\text{mCPBA}] - (k_7 + k_8 + k_9) \cdot [\text{compound II-mCPBA}] \quad (\text{Eq. 6})
\]

Experimentally determined values of the equilibrium and rate constants were assigned to the partial reactions defined in Scheme I. The kinetics of the intermediates and products defined in Schemes II, III, and IV (shown in Table I) were simulated using the corresponding differential equations.

**RESULTS AND DISCUSSION**

The Inactivation Pathway of HRP—Formation of verdohemoprotein (P670), with its characteristic absorption maximum at 670 nm, is diagnostic of peroxidase inactivation (19). The titration data shown in Fig. 1 with both spectrophotometric and enzyme activity monitoring show that at least two equivalents of mCPBA were required for its formation starting from HRP-C in the ferric state (Fig. 1). The loss of enzymatic activity is clearly proportional to the extent of P670 formation. These findings are consistent with the first equivalent of mCPBA being used to form compound I and the second to inactivate the enzyme and suggest that compound I is a common intermediate in the catalytic cycle and at the beginning of the inactivation pathway (Scheme III shown in Table I). It can also be calculated from Fig. 1 that 8 mol of mCPBA are needed to completely inactivate 1 mol of enzyme ([substrate]/[enzyme] ratio = 70

\[
[\text{compound II}] = k_3 [\text{compound I-mCPBA}] + k_4 \cdot [\text{compound II-mCPBA}] - k_5 \cdot [\text{compound II}] \quad (\text{Eq. 5})
\]

\[
[\text{compound II-mCPBA}] = k_6 [\text{compound II}][\text{mCPBA}] - (k_7 + k_8 + k_9) \cdot [\text{compound II-mCPBA}] \quad (\text{Eq. 6})
\]
The reaction of HRP-C with mCPBA is a diffusion-limited reaction (Dunford and Hewson (34)) to have a viscosity dependence characteristic of a diffusion limited reaction. The increase in absorbance at 670 nm is the maximum value reached in the reaction of 8.7 μM HRP-C with varying amounts of mCPBA in 10 mM sodium-phosphate buffer (pH 7.0), 25 °C. The residual enzyme activity was determined using the ABTS oxidation assay.

**Compound I Formation**—The reaction of HRP-C with a stoichiometric concentration of mCPBA yielded compound I with a UV-visible spectrum that was identical to that described for the reaction of this enzyme with hydrogen peroxide (23). No differences in the stability of this compound I were observed when compared with the compound I formed with hydrogen peroxide under the same conditions. The kinetics of the reaction of compound I with mCPBA under the same conditions ([mCPBA]₀ = 8.0). These equivalents are distributed between the catalytic cycle and the inactivation pathway (Scheme I).

**Scheme I. Reaction mechanism of mCPBA with HRP-C.** The species are as follows: E, native enzyme; compound I and compound II, active forms of the enzyme; Eᵢ (P-670), inactive form of the enzyme; mCBA, m-chlorobenzoic acid; R⁺, peroxyl radical of mCPBA.

mCPBA (8.7 μM = 8.0). These equivalents are distributed between the catalytic cycle and the inactivation pathway (Scheme I).

**Compound I Formation**—The reaction of HRP-C with a stoichiometric concentration of mCPBA yielded compound I with a UV-visible spectrum that was identical to that described for the reaction of this enzyme with hydrogen peroxide (23). No differences in the stability of this compound I were observed when compared with the compound I formed with hydrogen peroxide under the same conditions. The kinetics of the reaction of HRP-C with mCPBA to form compound I have been shown by Dunford and Hewson (34) to have a viscosity dependence characteristic of a diffusion limited reaction \(k₁ = 8.2 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) independent of pH in the range 5.0–6.8. Under single turnover conditions \([\text{HRP-C}]_0 > [\text{mCPBA}]_0\) (Fig. 2), we have determined that \(k₁ = (3.6 \pm 0.6) \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) at pH 7.0 (Table I). Under these conditions, no inactivation of the enzyme occurred nor was any verdohemoprotein formed.

**The Reaction of mCPBA with Compound I**—The two competing reactions that the complex formed between compound I and mCPBA undergoes (Scheme III shown in Table I) are proposed to explain the relationship between catalytic cycle and the inactivation pathways. In order to detect this transient intermediate and to determine the kinetics and mechanism of its formation and decay, the reaction of compound I with mCPBA was studied under stoichiometric conditions. Fig. 3A shows the formation of compound I-mCPBA intermediate and its subsequent decay to compound II and P670. The spectrum of the compound I-mCPBA complex was recorded after a 1-s reaction time and showed maxima at 411, 531, 556, and 649 nm and a shoulder at 615 nm (Fig. 3B). The extinction coefficient in the Soret region was calculated to be 45 mm⁻¹ cm⁻¹ using a global analysis with the computer program SPECFIT. A similar spectrum to that assigned to the compound I-mCPBA complex (Fig. 3B) was obtained in the reaction of compound I with m-chlorobenzoic acid, the parent carboxylic acid (not-peroxo form) of mCPBA (Fig. 4). This reaction when followed at 405 nm showed monophasic kinetics with a second-order rate constant of \(5.3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) and a dissociation constant of 0.25 mM. These results indicate that the complex formed between compound I and mCPBA is a real enzyme-substrate complex and could serve as a model for the HRP-C compound I-reducing substrate complex, which cannot be detected with conventional reducing substrates such as guaiacol or ABTS.

The enzyme species designated P940 and P670 have been observed previously in the reaction of HRP-C with an excess of hydrogen peroxide (19) and other peroxides (16–18). Another species, P965, has also been observed in the reaction of HRP-C with m-nitroperoxybenzoic acid (18). These authors proposed a sequential mechanism in which P965, formed directly from the reaction of compound I with m-nitroperoxybenzoic acid, subsequently reacted irreversibly with a second equivalent of the peroxide to give initially form P940 and then finally P670. In order to clarify the number of enzyme intermediates in the inactivation pathway, we have studied the reaction of compound I with mCPBA in the near infrared region (Fig. 5). We have been restricted to using conventional spectrophotometry with the first observation after manual mixing at ~15 s since our stopped-flow apparatus has a long wavelength limit of 700 nm. During the course of the reaction, an isosbestic point is maintained at 735 nm. The kinetics of the decrease in absorbance at 965 nm and the increase at 670 nm were identical and
were fitted to a single exponential function with $k_{\text{obs}} = 4 \times 10^{-3} \text{ s}^{-1}$ at 10°C, independent of the concentration of mCPBA (Fig. 5, inset). At the beginning, an increase in absorbance at 965 nm took place as consequence of the compound I-mCPBA complex formation. These data strongly suggest that the compound I-mCPBA complex is P965, which is the only intermediate that is detected when compound I is converted to verdohemoprotein (P670) by reaction with one equivalent of mCPBA. The similarity in the spectra of the complexes formed between compound I and both mCPBA and m-chlorobenzoic acid (Figs. 3B and 4, respectively) points to a chemical nature of P965 related to an unreacted enzyme-substrate intermediate whose formation is reversible, as was previously suggested for the (compound I-m-nitroperoxybenzoic acid) complex (18). The subsequent chemical structural change probably take place later, during the transition P965 → P670.

However, not all of the P965 is converted to P670 due to the competing decay reaction that yields compound II (Scheme III shown in Table I). Under single turnover conditions, with the first step equilibrating rapidly ($k_2[m\text{CPBA}] + k_{-2} \gg k_3, k_i$), the relaxation times are given by Equations 7 and 8.

$$
\lambda_1 = k_2[\text{compound I}] + k_{-2} \quad \text{(Eq. 7)}
$$

$$
\lambda_2 = \frac{k_{\text{max}}[\text{compound I}]}{(K_2 + [\text{compound I}])} \quad \text{(Eq. 8)}
$$

$K_2$ is the dissociation constant of the complex formed between compound I and mCPBA into compound I ($K_2 = k_{-2}k_2$) and $\lambda_{\text{max}}$ is $(k_3 + k_i)$. Equations 7 and 8 show that the faster relaxation time, $\lambda_1$, corresponds to the formation of the compound I-mCPBA complex and is independent of the slower process. However, the slower relaxation time, $\lambda_2$, is affected by the fast process (35). Under pseudo-first order conditions, with the concentration of compound I in large excess, the observed first-order rate constant for the formation of compound I-mCPBA at 411 nm exhibited a linear dependence with the concentration of compound I (Fig. 6). These data, together with Equation 7, allow the values of the elementary rate constant ($k_2$ and $k_{-2}$) and the dissociation constant ($K_2$) to be calculated (Table II). The formation of both compound II and verdohemoprotein were followed at 412 and 670 nm, respectively, under single turnover conditions (Fig. 7). Both species were formed with the same observed first-order rate constant, which was independent of the initial concentration of compound I, although the values of the actual first-order rate constants, $k_3$ and $k_i$, differ by a factor of 2 (see below). This surprising feature of the kinetics is predicted by the analysis given in the Appendix section and was confirmed by a series of simulations with different initial conditions, an example of which is shown in Fig. 7 (inset). The observed first-order rate constant is equal to $(k_3 + k_i)$ and has a value of $10^{-2} \text{ s}^{-1}$. Note that this last value
is similar to that obtained from Fig. 5B, because the process studied is the same, although at different temperature. The value of \( K_3 = 4.5 \times 10^{-6} \) m is significantly lower than the initial concentration of compound I and therefore \( \lambda_2 = \lambda_2 \text{max} \).

In order to determine the individual values of \( k_i \) and \( k_j \), we have measured the relative amounts of compound II and P670 formed under single turnover conditions as a function of increasing concentration of mCPBA (Fig. 8). The final concentration P670 was determined directly from the increase in absorbance at 670 nm using a calculated \( \epsilon_{670 \text{nm}} = (19.6 \pm 2.0) \text{mM}^{-1} \text{cm}^{-1} \). The final concentration of compound II could not be measured spectrophotometrically since several forms of the enzyme absorb in the Soret region. Instead, it was estimated from the remaining enzymatic activity, using the ABTS oxidation assay. The data in Fig. 8 show that both determinations give a good linear correlation, indicating that the distribution of compound I-mCPBA into compound II (67 ± 3%) and P670 (33 ± 2%) was independent of the initial concentration of the complex. This distribution can be defined as the partition ratio \( r = k_g/k_i = 2.0 \) and allows the values of \( k_g \) and \( k_i \) to be calculated in good agreement with the values obtained previously under pseudo-steady-state conditions (Table II) (26).

In addition, our titration data (Fig. 1) show that 8 mol of mCPBA were necessary to completely inactivate 1 mol of enzyme. According to the calculated partition ratio of 2, the reaction mechanism depicted in Scheme I is consistent with this stoichiometry. Additional consumption of mCPBA in the inactivation pathway would result in a different titration value. In consequence, only one molecule of mCPBA is required to react with compound I for some inactivation to be observed, and this is not consistent with other mechanisms suggesting that two molecules of hydroperoxide from compound I are needed in order to convert the enzyme to inactive P670 state (18).

The Reaction of mCPBA with Compound II—An inactivation pathway originating with compound II has been proposed previously (19). In order to further investigate this proposal, we have studied the reaction of compound II with stoichiometric and excess concentrations of mCPBA using conventional diode-array and stopped-flow spectrophotometry. Compound II with characteristic absorption maxima at 417, 529, and 556 nm was generated from compound I by the addition of one equivalent of ascorbic acid. When compound II reacted with one equivalent of mCPBA, a transient spectrum of an intermediate (assumed to be the compound II-mCPBA complex) was initially observed. This spectrum subsequently converted to that characteristic of the native ferric state with an isosbestic point maintained at 411 nm (Fig. 9). The failure to detect any P670 product strongly suggests that only compound I and not compound II is involved in the initiation of the inactivation reaction. Under these conditions and with a 10-fold excess of mCPBA, no spectroscopic evidence for transient compound III formation was obtained. Thus the ferric enzyme product is probably formed by single electron transfer from mCPBA to compound II.

The kinetics of the reaction of compound II with mCPBA were studied under single turnover conditions with increasing concentrations of compound II at a fixed substoichiometric concentration of mCPBA. The time dependence of the absorbance change at 403 nm showed an initial decrease with a minimum at 25 s that corresponds to the formation of the transient intermediate, compound II-mCPBA. This was followed by an increase in the absorbance due to the formation of native ferric enzyme. Assuming compound II-mCPBA complex formation occurs as a rapid pre-equilibrium prior to electron transfer, \( (k_i[\text{mCPBA}] + k_{-4} \gg k_g) \), the observed first order rate constants for the two reactions, \( \lambda_1' \) and \( \lambda_2' \), are given by Equations 9 and 10 (35).

\[
\lambda_1' = k_g[\text{compound II}] + k_{-4} \quad (\text{Eq. 9})
\]

\[
\lambda_2' = \lambda_2 \text{max}[\text{compound II}]/(K_4 + [\text{compound II}]) \quad (\text{Eq. 10})
\]

\[\lambda_2' = k_5 \text{and } K_4 = k_{-4}/k_4. \]

Fig. 10 shows the linear dependence of \( \lambda_1' \) on the concentration of compound II under pseudo-first-order conditions ([compound II], \( \gg [\text{mCPBA}] \)). The slope and intercept give the values of \( k_g, k_{-4}, \) and \( K_4 \) (Table II). The electron transfer from mCPBA to compound II within the complex was very slow \( \lambda_2' = 1.0 \times 10^{-3} \text{ s}^{-1} \), as shown by the kinetics of the appearance of native ferric enzyme with monitoring at 403 nm (data not shown).

![Fig. 6. Linear plot of the second-order rate constant, \( k_{\text{obs}} \) versus the initial concentration of compound I for the reaction with 0.5 \( \mu \)M mCPBA under single turnover conditions in 10 mM sodium-phosphate buffer (pH 7.0), 25°C.](image)

**Table II**

Kinetic constants which characterized the inactivation of HRP-C by mCPBA

| Catalytic step | Pre-steady-state | Pseudo-steady-state |
|---------------|------------------|---------------------|
| \( E + \text{mCPBA} \rightarrow \text{CoI} \) | \( k_1 \text{ (m}^{-1}\text{s}^{-1}) = (3.6 \pm 0.6) \times 10^7 \) | \( K_4 \text{ (m) = (4.5 \pm 0.5) \times 10^{-6} \) |
| \( \text{CoI} + \text{mCPBA} \rightarrow [\text{CoI-mCPBA}] \) | \( k_2 \text{ (m}^{-1}\text{s}^{-1}) = (1.1 \pm 0.1) \times 10^6 \) | \( k_3 \text{ (s}^{-1}) = (6.4 \pm 0.1) \times 10^{-3} \) |
| \( [\text{CoI-mCPBA}] \rightarrow \text{CoII} \) | \( k_2 \text{ (s}^{-1}) = (3.3 \pm 0.1) \times 10^{-3} \) | \( k_3 \text{ (s}^{-1}) = (4.8 \pm 0.4) \times 10^{-3} \) |
| \( \text{CoII} + \text{mCPBA} \rightarrow [\text{CoII-mCPBA}] \) | \( k_4 \text{ (m}^{-1}\text{s}^{-1}) = (3.5 \pm 0.1) \times 10^6 \) | \( K_4 \text{ (m) = (5.7 \pm 0.5) \times 10^{-7} \) |
| \( [\text{CoII-mCPBA}] \rightarrow \text{E} \) | \( k_4 \text{ (s}^{-1}) = (2.2 \pm 0.2) \times 10^{-3} \) | \( k_3 \text{ (s}^{-1}) = (1.5 \pm 0.1) \times 10^{-3} \) |
| Partition ratio \( r \) | \( k_2/k_1 = (2.0 \pm 0.1) \) | \( k_3/k_4 = (1.8 \pm 0.1) \) |

\footnote{This work.}

\footnote{Ref. 26.}
shown). Under these conditions, the low value of $K_4 = 5.7 \times 10^{-7}$ M results in $\lambda_{12}$ being independent of compound II concentration and equal to $k_5$. This value of $k_5$ is in agreement with that obtained under steady-state conditions with ($[\text{mCPBA}]_0 \gg [\text{HRP-C}]_0$) (Table II) (26).

CONCLUSIONS

The experiments described above have shown conclusively that mCPBA induced inactivation of HRP-C occurs by the mechanism described in Scheme I. The data are not consistent with the mechanism proposed by Marklund (17) nor with the modified mechanism of Nakajima and Yamazaki (18). Our kinetic and titration data clearly demonstrate that only two molecules of mCPBA are necessary to form inactive verdohe- moprotein from the native ferric state of HRP-C. In addition, other mechanisms that have invoked an inactivation pathway originating at the compound II state (19, 36) are also not consistent with our data. No inactivation of the heme was observed when mCPBA reacted with an excess of compound II.

(i) The peroxidase inactivation pathway originates at a transient intermediate formed during the reaction of compound I with the hydroperoxide. This intermediate (P965) can be considered to be a model for the binding of reducing substrates to compound I and has spectral similarities to those of a complex formed between $m$-chlorobenzoic acid and compound I.

(ii) This intermediate decays in competing reactions to give compound II (67%) and an inactive form of HRP-C, P670 (33%) with mCPBA. The product ratio is determined only by the
relative values of the inactivation and the catalytic rate constants.

(iii) The active center in different class III peroxidases appears to be well conserved (1), and the role of amino acids such as His-170, His-42, and Arg-38 is well established for HRP-C (11, 37). Apparently, this active center architecture is optimized for compound I formation, although with excess hydroperoxide inactivation occurs. Consequently, mutations in Arg-38 (22) or His-42 (37) make the variants more susceptible to inactivation by hydroperoxides. However, when conventional reducing substrates such as ABTS or guaiacol are present, the extent of inactivation is decreased since the steady-state level of compound I, which reacts with the hydroperoxide to initiate inactivation, is much lower, i.e., reducing substrate protects the enzyme from inactivation by removing compound I (38, 39).

(iv) It is interesting, from a physiological perspective, to point out that HRP-C shows a high affinity for hydroperoxides, shows a high rate of compound I formation, and is active at low concentrations of reducing substrate. However, it is more sensitive to inactivation under these conditions (21). These properties would allow HRP-C to act as an efficient detoxifying system for the elimination of excess hydroperoxide, providing the nature and concentration of reducing substrate ensures a competent rate of compound II reduction to ferric enzyme in the catalytic cycle. The danger of accumulating deleterious free radical could thus be avoided by a coupled process involving HRP reaction products and an antioxidant (i.e., ascorbic acid) (40, 41). However, if there is a perturbation in the cell status toward more oxidative conditions (i.e., the H₂O₂ burst induced by pathogenesis), then the detoxifying capacity of the enzyme could be surpassed, its susceptibility to inactivation would increase (at higher oxidant/reductant ratios), and the damaging free radical would not be reduced. Chain reactions could also result which can also contribute to cell death in the so-called "hypersensitive response" (42). The effect of H₂O₂ and mCPBA in vivo have recently been demonstrated by modifications in the patterns of growth and morphogenesis of lupin hypocotyls after application of different concentrations of these hydroperoxides (43).

The use of pre-steady-state kinetics under single turnover conditions has not only confirmed the values of the limited number of combined rate and equilibrium constants that we have previously determined under pseudo-steady-state conditions with mCPBA in large excess (26), but has also allowed the determination of the elementary rate constants for the partial reactions that comprise the mechanism of inactivation. In addition the UV-visible spectrum of the kinetically competent, the extent of inactivation is decreased since the steady-state level of compound I, which reacts with the hydroperoxide to initiate inactivation, is much lower, i.e., reducing substrate protects the enzyme from inactivation by removing compound I (38, 39).

The proposed mechanism for the reaction of compound I with mCPBA is depicted in Scheme III (shown in Table I). This mechanism is a modification of a two-step mechanism (35) in which compound I is stable but mCPBA induces instability of the complex compound I-mCPBA (46). The time-course equations of the concentrations of compound II and inactive enzyme (P670) under single turnover are given for the following expressions.

\[
\text{[compound II]} = a_I \text{[compound I]} + \frac{\beta_1}{\lambda_1(\lambda_1 - \lambda_2)} e^{-\lambda_1 t} + \frac{\beta_1}{\lambda_1(\lambda_2 - \lambda_1)} e^{-\lambda_2 t} \quad \text{(Eq. 1A)}
\]

\[
[E_I] = a_{I2} \text{[compound I]} + \frac{\beta_2}{\lambda_1(\lambda_1 - \lambda_3)} e^{-\lambda_1 t} + \frac{\beta_2}{\lambda_3(\lambda_3 - \lambda_1)} e^{-\lambda_3 t} \quad \text{(Eq. 2A)}
\]

In Equations 1A and 2A, the terms are defined as shown by Equations 3A–6A below.

\[
a_1 = k/k_1 + k_i \quad \text{(Eq. 3A)}
\]

\[
a_2 = k/k_1 + k_i \quad \text{(Eq. 4A)}
\]

\[
\beta_1 = k_i k_1 \text{[compound I][mCPBA]} \quad \text{(Eq. 5A)}
\]

\[
\beta_2 = k_i k_2 \text{[compound I][mCPBA]} \quad \text{(Eq. 6A)}
\]

The values for \(\lambda_1\) and \(\lambda_2\) are given by Equations 7A and 8A.

\[
\lambda_1 = \frac{1}{2} \left[ k + \sqrt{(k)^2 - 4lk} \right] \quad \text{(Eq. 7A)}
\]

\[
\lambda_2 = \frac{1}{2} \left[ k - \sqrt{(k)^2 - 4lk} \right] \quad \text{(Eq. 8A)}
\]

For Equations 7A and 8A, terms are defined in Equations 9A and 10A.

\[
\sum k = k_1 \text{[compound I]} + k_2 + k_3 + k_i \quad \text{(Eq. 9A)}
\]

\[
P_{1k} = k_i k_2 + k_i \text{[compound I]} \quad \text{(Eq. 10A)}
\]

From Equations 7A through 10A, we have the following relationships for the sum and product of the two relaxation times.

\[
\lambda_1 + \lambda_2 = k_2 \text{[compound I]} + k_1 + k_3 + k_i \quad \text{(Eq. 11A)}
\]

\[
\lambda_1 \lambda_2 = k_i k_3 + k_i \text{[compound I]} \quad \text{(Eq. 12A)}
\]

We have observed that the bimolecular process is sufficiently faster that both unimolecular process, or, more strictly, the following inequality holds.

\[
k_i \text{[compound I]} + k_{1+2} \gg k_i \text{[compound I]} \quad \text{(Eq. 13A)}
\]

In this case, the two relaxation times are well separated. The faster process, \(\lambda_1\), is given by Equation 14A and corresponds to Equation 7 under "Results and Discussion."

\[
\lambda_1 = k_i \text{[compound I]} + k_{1+2} \quad \text{(Eq. 14A)}
\]

The longer relaxation time or slower process, \(\lambda_2\), can be obtained by substituting Equation 14A into Equation 12A, to obtain Equation 15A, as follows.

\[
\lambda_2 = \frac{k_3 + k_i \text{[compound I]}}{K_2 + \text{[compound I]}} \quad \text{(Eq. 15A)}
\]

\(K_2\) is the dissociation constant of the complex compound I-mCPBA into compound I and mCPBA (\(K_2 = k_{1+2} / k_i \)).

Therefore, the real time courses for compound II and P670 are given by Equations 16A and 17A.

\[
\text{[compound III]} = a_{I3} \text{[compound I]}(1 - e^{-\lambda_1 t}) \quad \text{(Eq. 16A)}
\]

\[
[E] = a_{I2} \text{[compound I]}(1 - e^{-\lambda_2 t}) \quad \text{(Eq. 17A)}
\]

The experimental traces obtained at 412 and 670 nm with \([\text{compound I}] \gg K_2\) which correspond to the accumulation of compound II and verdohemoprotein, respectively, fit to
Equations 16A and 17A with the same observed rate constant \( (k_2 + k_1) \).

A similar procedure can be followed to obtain the equation for the reaction between compound II and mCPBA. In this case the first order rate constants are named \( \lambda_1 \) and \( \lambda_2 \) respectively (see Equations 9 and 10, under “Results and Discussion”).

REFERENCES
1. Penel, C., Gaspar, T. H., and Greppin, H. (eds) (1992) *Plant Peroxidases: 1980–1990*, University of Geneva, Geneva, Switzerland
2. Shannon, L. M., Kay, E., and Lew, J. Y. (1966) *J. Biol. Chem.* 241, 2166–2172
3. Dunford, H. B., and Stillman, J. S. (1976) *Coord. Chem. Rev.* 19, 187–251
4. Yamazaki, I., Tamura, M., and Nakajima, R. (1981) *J. Biol. Chem.* 256, 13325–13343
5. Dunford, H. B. (1991) *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., eds) Vol. II, pp. 1–24, CRC Press, Boca Raton, FL
6. Marañón, M. J. R., and Van Huystee, R. B. (1994) *Phytochemistry* 37, 1217–1225
7. Shiro, Y., Kurono, M., and Morishima, I. (1986) *J. Biol. Chem.* 261, 9382–9390
8. Morishima, I., Kurono, M., and Shiro, Y. (1986) *J. Biol. Chem.* 261, 9391–9399
9. Smith, A. T., Santama, N., Dacey, S., Bray, R. C., Thorneley, R. N. F., and Burke, J. F. (1990) *J. Biol. Chem.* 265, 13325–13343
10. Baeck, H. K., and Van Wart, H. E. (1992) *J. Am. Chem. Soc.* 114, 718–725
11. Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) *J. Biol. Chem.* 271, 4023–4030
12. Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) *J. Biol. Inorg. Chem.* 1, 136–142
13. Keilin, D., and Hartree, E. F. (1951) *Biochem. J.* 49, 88–104
14. Nicholls, P., and Schonbaum, G. R. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H., and Myrbach, K., eds) Vol. 8, pp. 47–225, Academic Press, New York
15. Nakajima, R., and Yamazaki, I. (1987) *J. Biol. Chem.* 262, 2576–2581
16. Chance, B. (1949) *Arch. Biochem.* 21, 416–430
17. Marklund, S. (1975) *Arch. Biochem. Biophys.* 154, 614–622
18. Nakajima, R., and Yamazaki, I. (1980) *J. Biol. Chem.* 255, 2067–2071
19. Bagger, S., and Williams, R. J. P. (1971) *Acta Chem Scand.* 25, 976–982
20. Arnao, M. B., Arcosta, M., Del Río, J. A., Varoñ, R., and García-Cánovas, F. (1990) *Biochim. Biophys. Acta* 1041, 43–47
21. Hiner, A. N. P., Hernández-Ruiz, J., García-Cánovas, F., Arnao, M. B., and Acosta, M. (1996) *Biotech. Bioeng.* 50, 655–662
22. Hiner, A. N. P., Hernández-Ruiz, J., García-Cánovas, F., Smith, A. T., Arnao, M. B., and Acosta, M. (1995) *Eur. J. Biochem.* 234, 506–512
23. Schonbaun, G. R., and Lo, S. (1972) *J. Biol. Chem.* 247, 3353–3360
24. Davies, D. M., Jones, P., and Mantle, D. (1976) *Biochem. J.* 157, 247–253
25. Joh, D., and Jones, P. (1978) *Eur. J. Biochem.* 86, 565–567
26. Arnao, M. B., Hernández-Ruiz, J., Varoñ, R., García-Cánovas, F., and Acosta, M. (1995) *J. Mol. Catal. A Chem.* 104, 179–191
27. Acosta, M., Del Río, J. A., Arnao, M. B., Sánchez-Bravo, J., Sabater, F., García-Carmona, F., and García-Cánovas, F. (1988) *Biochim. Biophys. Acta* 955, 194–202
28. Gazaryan, I. G., Lagrimini, M., Ashby, G. A., and Thorneley, R. N. F. (1996) *Biochem. J.* 322, 841–847
29. Chamoun, W., Takahashi, N., and Mason, R. P. (1989) *J. Biol. Chem.* 264, 7899–7909
30. Acosta, M., Arnao, M. B., Hernández-Ruiz, J., and García-Cánovas, F. (1993) in *Plant Peroxidases: Biochemistry and Physiology* (Weinnder, K. G., Rasmussen, S. K., Penel, C., and Greppin, H., eds) pp. 201–205, University of Geneva, Geneva, Switzerland
31. Bielski, B. H. D., and Allen, A. O. (1977) *J. Phys. Chem.* 81, 1048–1050
32. Childs, R. E., and Bardsley, W. G. (1975) *Biochem. J.* 145, 93–103
33. Hiromi, K. (1979) *Kinetcs of Fast Enzyme Reactions*, pp. 244–247, Halsted Press, New York
34. Dunford, H. B., and Hewson, W. D. (1977) *Biochemistry* 16, 2849–2857
35. Bernasconi, C. F. (1976) *Relaxation Kinetics*, pp. 31–34, Academic Press, New York
36. Adediran, S. A., and Lambeir, A. (1989) *Eur. J. Biochem.* 186, 571–576
37. Newyarger, S. L., and Ortiz de Montellano, P. R. (1995) *J. Biol. Chem.* 270, 19430–19438
38. Arnao, M. B., Acosta, M., Del Río, J. A., and García-Cánovas, F. (1990) *Biochim. Biophys. Acta* 1038, 85–89
39. Arnao, M. B., García-Cánovas, F., and Acosta, M. (1996) *Biochem. Mol. Biol. Int.* 39, 97–107
40. Takahama, U. (1993) *Physiol. Plant.* 89, 791–798
41. Mehlhorn, H., Lelandais, M., Kerth, H. G., and Poyer, C. H. (1996) *FEBS Lett.* 378, 203–206
42. Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994) *Cell* 79, 583–593
43. Cano, A., Artes, F., Arnao, M. B., Sánchez-Bravo, J., and Acosta, M. (1996) *J. Plant Physiol.* 147, 721–728
44. Miller, V. P., DePillis, G. D., Ferrer, J. C., Mauk, A. G., and Ortiz de Montellano, P. R. (1992) *J. Biol. Chem.* 267, 8936–8942
45. Gilfoyle, D. J., Rodriguez-Lopes, J. N., and Smith, A. T. (1996) *Eur. J. Biochem.* 236, 714–722
46. Garrido-del Soló, C., García-Cánovas, F., Havsteen, B. H., and Varon, R. (1993) *Biochem. J.* 294, 459–464