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Short Communication

Complexes Between m-chloroperoxybenzoic Acid and Horseradish Peroxidase Compounds I and II: Implications for the Kinetics of Enzyme Inactivation

ALEXANDER N.P. HINER[a,*], JOSEFA HERNÁNDEZ RUIZ[b], MARINO B. ARNAO[b], JOSE NEPTUNO RODRÍGUEZ LÓPEZ[a], FRANCISCO GARCÍA CÁNOVAS[a] and MANUEL ACOSTA[b]

[a]*GENZ: Grupo de Investigación de Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología (Edificio de Veterinaria), Universidad de Murcia, E-30100 Espinardo, (Murcia) Spain;* [b]*Departamento de Biología Vegetal (Fisiología Vegetal), Facultad de Biología, Universidad de Murcia, E-30100 Espinardo, (Murcia) Spain*

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INTRODUCTION

Horseradish peroxidase (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7.) (HRP) is the best known and most studied[1,2] member of class III[3] of the widely distributed superfamily of haem peroxidases from plants, fungi and prokaryotes. More than 30 isoenzymes of HRP have been identified but the slightly basic isoenzyme C (HRP-C) is the most abundant form.[4]

The catalytic cycle of HRP-C is well established.[5] The resting enzyme, containing a single ferric haem prosthetic group (oxidation state +3), undergoes a rapid ($k_1 > 10^7\,M^{-1}\,s^{-1}$) reaction with a hydroperoxide (H_2O_2 or an organic peroxide) to give an oxidized intermediate termed compound I [oxyferryl iron (Fe(IV) = O) and porphyrin π-cation radical, oxidation state +5].[6] The ferric enzyme is usually recovered in two single electron reduction steps with a suitable donor substrate,[5] yielding free radical products. After the first of these steps a second intermediate compound II (oxyferryl iron, oxidation state +4) is detected.

A few donor substrates have been identified that are capable of the direct regeneration of ferric HRP-C in a single two-electron reduction step.[7] One such substrate is in fact H_2O_2, releasing oxygen gas in a catalase-like reaction.[8–10] H_2O_2 will also react with compound I as a one-electron donor releasing superoxide radicals.[8–10] However, the turnover of HRP-C with H_2O_2 via these two routes is accompanied by (and is in competition with) the progressive loss of enzymatic activity due to the action of H_2O_2 as a suicide (mechanism-based) inactivator.[9,11–13]

The xenobiotic hydroperoxide m-chloroperoxybenzoic acid (mCPBA) possesses high affinity for HRP-C, the reaction to form compound I proceeds more rapidly than in the case of H_2O_2, and it will act as a one-electron donor to compound I. mCPBA is also a very much more potent inactivating agent than H_2O_2 due to the lack of a catalase-like two-electron reduction reaction. Comprehensive steady

*Corresponding author. Address: GENZ: Grupo de Investigación de Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología (Edificio de Veterinaria), Universidad de Murcia, E-30100 Espinardo, (Murcia) Spain. Tel.: +34-968-228283. Fax: +34-968-364147. E-mail: ahiner@um.es

Abbreviations: HRP-C, horseradish peroxidase isoenzyme C; CI, compound I; CII, compound II; mCPBA, m-chloroperoxybenzoic acid; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid).

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state and transient state kinetic and spectroscopic studies of the activity of mCPBA with HRP-C[14–16] have identified the reaction intermediates and the rate constants controlling their evolution; these data are summarized by the mechanistic scheme shown in Fig. 1. A point of central importance in the mechanism is the formation of the complex between compound I and mCPBA (CI·mCPBA), from which a partition occurs between catalytic turnover via compound II (CII) back to ferric HRP-C (E), and enzyme inactivation (E_i). The relative proportion of each reaction is described by the partition ratio, $r = k_3/k_i$, experimentally determined to have a value of 2.[16] Thus, some 33% of the total enzyme activity is lost each time a catalytic cycle is completed, until HRP-C is completely inactivated or the mCPBA is exhausted.

Despite the determination of all the controlling rate constants, an aspect of the reaction of HRP-C with mCPBA has remained unclear. The value of the inactivation constant, k_i, accurately measured using the single turnover stopped-flow method,[16] was not sufficient to justify the velocity of inactivation observed as the fall in peroxidase activity in a standard assay system comprising H_2O_2 and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). We have therefore further examined the reaction kinetics and used computer simulations of the scheme (Figure 1) to seek an explanation of this effect.

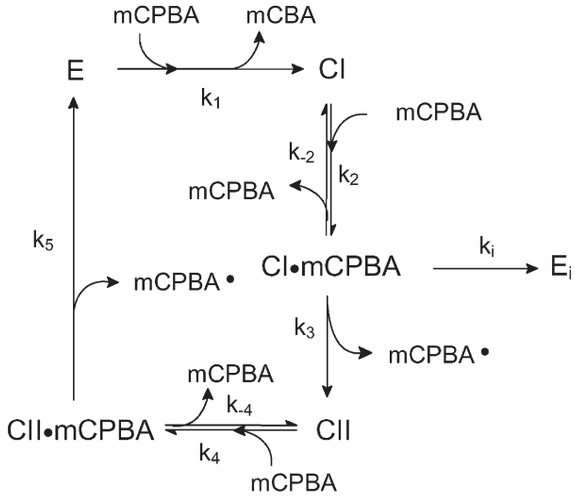

FIGURE 1 Scheme showing the reactions of horseradish peroxidase with m-chloroperoxybenzoic acid. E is ferric HRP-C; mCPBA is m-chloroperoxybenzoic acid; mCPBA• is the radical of mCPBA; mCBA is m-chlorobenzoic acid; CI and CI·mCPBA, CII and CII·mCPBA are compounds I and II and their respective complexes with mCPBA; E_i is inactivated HRP-C (P670). In the scheme HRP-C exhibits a catalytic and an inactivation pathway from the CI·mCPBA complex. During catalytic turnover free radical products are generated by one electron oxidation of the substrate, mCPBA., to recover ferric HRP-C. Inactivation results in the formation of verdohemochrome P670 followed by loss of the haem moiety, leaving colourless products. The proportion of enzyme that follows each route is described by the partition ratio $r = k_3/k_i$, the value of r is 2.[16] Thus <5 turnover cycles with m-CPBA result in the virtually complete suppression of enzyme activity.

MATERIALS AND METHODS

Materials

HRP-C, $A_{403\,nm}/A_{275\,nm}(RZ) = 3.2$, was obtained from Biozyme Laboratories (product code HRP-4B). This preparation has been confirmed to be iso-enzyme C using isoelectric focussing (single band with pI 8.5).[13] Enzyme concentrations were determined spectrophotometrically using $\epsilon_{403\,nm} = 100\,mM^{-1}\,cm^{-1}$. mCPBA (≈ 80% pure) was obtained from Aldrich and further purified by recrystallization from 40–60° light petroleum ether/diethylether (3:1 v/v) following the protocol of Davies *et al.*[17] to give an ≈ 99% pure product (from NMR analysis and a sharp melting point at 92–94°C). Stock solutions of mCPBA were made up in 1:1 v/v ethanol/water immediately prior to use. The concentration was estimated using $\epsilon_{232\,nm} = 8940\,M^{-1}\,cm^{-1}$. Hydrogen peroxide (30% by vol.) was from Sigma and its concentration was determined using $\epsilon_{240\,nm} = 43.6\,M^{-1}\,cm^{-1}$. ABTS was purchased from Sigma and its concentration measured spectrophotometrically using $\epsilon_{340\,nm} = 36\,mM^{-1}\,cm^{-1}$. Buffer substances (analytical reagent grade) were obtained from Merck. All solutions were prepared using de-ionized water drawn from a Milli-Q system (Millipore).

Kinetics of HRP-C Inactivation by MCPBA

The inactivation of HRP-C was followed against time at pH 6.5 (50 mM sodium phosphate buffer) as the fall in peroxidase activity. HRP-C (1 μM) was incubated with mCPBA (10, 20, 50 and 100 μM). At appropriate times after the start of reaction, aliquots (10 μL) were removed and the activity with ABTS was determined at 414 nm (radical product, $\epsilon = 31.1\,mM^{-1}\,cm^{-1}$). The activity assay comprised ABTS (1 mM), H_2O_2 (0.2 mM) in 50 mM sodium citrate buffer, pH 4.5 in a total volume of 1 mL. In some ABTS, oxidation assays higher H_2O_2 concentrations (1, 10, 50 mM) were used. The residual activities (%) were calculated for each time point and mCPBA concentration. Assays were recorded on a Perkin-Elmer Lambda-2S UV-vis spectrophotometer. The temperature was controlled at 25 ± 0.1°C using a Haake D1G circulating water bath. Oxygen production was measured using a Clark-type electrode coupled to an oxygraph system (Hansatech, Kings Lynn, Cambs., UK) as previously described.[9,10]

Computer Simulation

The scheme (Figure 1) for the reaction of HRP-C with mCPBA was used as the basis for computer simulations using the programs Ksim (v. 1.1 by N. C. Millar) or Wes (v. 1.0 by F. García Sevilla *et al.*).[18] Similar results were obtained using either program.

The reaction proceeded in the transition phase until the mCPBA was exhausted or the enzyme was fully inactivated. The differential rate equations that describe the concentrations of each species in the kinetic model are as follows:

Ferric HRP-C.

$$\frac{d[E]}{dt} = k_5[CII{\cdot}mCPBA] - k_1[mCPBA][E] \quad (1)$$

Compound I.

$$\frac{d[CI]}{dt} = k_1[mCPBA][E] + k_{-2}[CI{\cdot}mCPBA] - k_2[CI][mCPBA] \quad (2)$$

Compound I·mCPBA complex.

$$\frac{d[CI{\cdot}mCPBA]}{dt} = k_2[CI][mCPBA] - (k_i + k_3 + k_{-2})[CI{\cdot}mCPBA] \quad (3)$$

Compound II.

$$\frac{d[CII]}{dt} = k_3[CI{\cdot}mCPBA] + k_{-4}[CII{\cdot}mCPBA] - k_4[mCPBA][CII] \quad (4)$$

Compound II·mCPBA complex.

$$\frac{d[CII{\cdot}mCPBA]}{dt} = k_4[mCPBA][CII] - (k_{-4} + k_5) \times [CII{\cdot}mCPBA] \quad (5)$$

Inactivated enzyme.

$$\frac{d[E_i]}{dt} = k_i[CI{\cdot}mCPBA] \quad (6)$$

m-Chloroperoxybenzoic acid free radical.

$$\frac{d[mCPBA^{\cdot}]}{dt} = k_3[CI{\cdot}mCPBA] + k_5[CII{\cdot}mCPBA] \quad (7)$$

m-Chlorobenzoic acid.

$$\frac{d[mCBA]}{dt} = k_1[mCPBA][E] \quad (8)$$

m-Chloroperoxybenzoic acid.

$$\frac{d[mCPBA]}{dt} = k_{-2}[CI{\cdot}mCPBA] + k_{-4}[CII{\cdot}mCPBA] - (k_1[mCPBA][E] + k_2[mCPBA] \times [CI] + k_4[mCPBA][CII]) \quad (9)$$

Where E is ferric HRP-C, CI and CII are compounds I and II, respectively, CI·mCPBA and CII·mCPBA are the complexes of mCPBA with compounds I and II, respectively, E_i is inactivated HRP-C (verdohemochrome P670), mCPBA is m-chloroperoxybenzoic acid, mCPBA$^{\cdot}$ is the free radical of mCPBA, and mCBA is m-chlorobenzoic acid.

The values of the rate constants used in the simulation have previously been determined experimentally[16] and were as follows: $k_1 = 3.6 \times 10^7\,M^{-1}\,s^{-1}$, $k_2 = 1.1 \times 10^6\,M^{-1}\,s^{-1}$, $k_{-2} = 4.9\,s^{-1}$ ($K_2 = 4.5 \times 10^{-6}\,M$), $k_3 = 6.4 \times 10^{-3}\,s^{-1}$, $k_4 = 3.5 \times 10^5\,M^{-1}\,s^{-1}$, $k_{-4} = 2.2 \times 10^{-1}\,s^{-1}$, ($K_4 = 5.7 \times 10^{-7}\,M$), $k_5 = 1.0 \times 10^{-3}\,s^{-1}$ and $k_i = 3.3 \times 10^{-3}\,s^{-1}$.

RESULTS AND DISCUSSION

Figure 2A shows the time courses of inactivation of HRP-C (1 μM) by 10, 20 and 100 molar equivalents of mCPBA (i.e. 10, 20 and 100 μM) determined as the fall in peroxidase activity in assays with H_2O_2 (0.2 mM) and ABTS (1 mM). The observed rates of inactivation were clearly dependent on the mCPBA concentration and have previously[15] been used to calculate a value of the inactivation constant (k_i) of $4.8 \times 10^{-3}\,s^{-1}$ in reasonable agreement with the more reliable value ($k_i = 3.3 \times 10^{-3}\,s^{-1}$) obtained from direct spectroscopic observations of the formation of inactivated enzyme (E_i) in single turnover stopped-flow experiments.[16]

This experimental method has also been used to determine all the other rate constants in the mechanism shown in Figure 1; it was therefore possible to accurately simulate the reaction (see Computer simulation). The simulation results in Figure 2B show the concentrations of the complexes compound I·mCPBA (CI·mCPBA) and compound II·mCPBA (CII·mCPBA), and E_i for the reaction of HRP-C (1 μM) with 10 equivalents of mCPBA. The simulated concentration curve for E_i clearly indicates that its formation was not sufficient to justify the experimental inactivation profile from Figure 2A. This was also observed when higher concentrations (20 or 100 μM) of mCPBA were simulated (not shown). In these cases the concentration curves for E_i were almost identical to that for 10 μM and thus did not exhibit the concentration dependence seen in

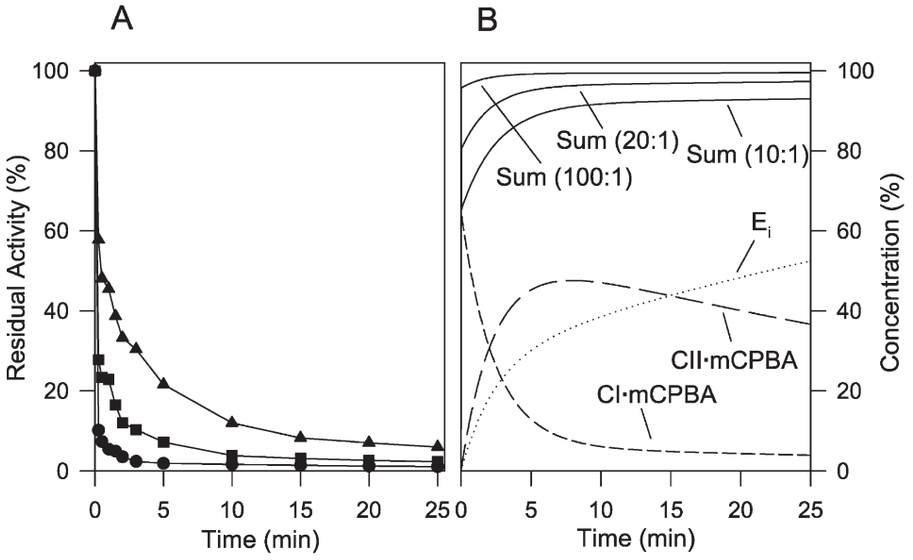

FIGURE 2 (A) Time courses of inactivation of horseradish peroxidase with m-chloroperoxybenozoic acid. HRP-C (1 μM) was incubated with mCPBA (▲, 10 μM; ■, 20 μM; ●, 100 μM) and at the appropriate times aliquots were taken and the residual activity with H_2O_2/ABTS was determined (see text). (B) Simulation of the scheme in Figure 1. The simulated conditions were HRP-C (1 μM) with mCPBA (10 μM) showing the concentrations (%) of the complexes of compounds I and II with mCPBA (CI·mCPBA and CII·mCPBA, respectively), inactivated HRP-C (E_i), and the sum of the concentrations of these three species (Sum 10:1). The sums of the species in the cases where 20 or 100 μM mCPBA were simulated are also shown (Sum 20:1 and Sum 100:1 respectively).

experiments. The main difference at 100 compared to 10 equivalents of mCPBA was that, with more mCPBA, a higher concentration (approaching 100%) of CI·mCPBA was formed at the beginning of the reaction.

It is reasonable to expect that native peroxidase (E) and compounds I and II (CI and CII) will be active under standard assay conditions since these species participate in the normal catalytic cycle of peroxidase (see Introduction). Taking the remaining enzymatic species involved in the mechanism (Figure 1) it was found that when the sums of the concentrations of the two complexes plus E_i were plotted in simulations of 10, 20 and 100 molar equivalents of mCPBA (Figure 2B) this resulted in much better agreement with the experimental curves at each concentration. The rapid initial fall in activity appeared to be mainly due to the formation of CI·mCPBA and the subsequent curve shape was the result of the evolving composition of the mixture of the three species. Simulations of longer time periods (not shown) indicated that the true reaction end-point was reached after several hours when a mixture of E_i along with a small amount of native peroxidase, which accounted for any residual activity, remained. It is known that the affinities of mCPBA for HRP-C compounds I and II are around three orders of magnitude higher than the respective values with H_2O_2[15,16] (see Computer simulation). Therefore, it appeared that the complexes (CI·mCPBA and CII·mCPBA) were equivalent to inactivated enzyme with regard to their activity in ABTS oxidation assays presumably due to the much higher affinity of mCPBA for the compounds of HRP-C compared to the added H_2O_2.

In order to test this hypothesis ABTS oxidation assays were performed with mCPBA-inactivated HRP-C (15-minute incubation) using higher H_2O_2 concentrations (1, 10 and 50 mM) than the usual (0.2 mM). The data for 10 and 50 mM H_2O_2 (not shown) exhibited the normal level of activity at the start but then the rate of ABTS oxidation was observed to rise, after approximately one minute, to a value approximating that expected from the simulated concentration of E_i. The rates of ABTS oxidation using 0.2 and 1 mM H_2O_2 remained constant throughout the assay period of up to 10 minutes. These data can be explained if the higher concentrations of H_2O_2 could displace mCPBA from the enzyme active site. The low dissociation rates of mCPBA from the complexes with HRP-C and the low rates of evolution of the complexes (i.e. CI·mCPBA to compound II and CII·mCPBA to ferric HRP-C) accounted for the initial lower ABTS oxidation rate.

An additional consideration when H_2O_2 was added in large excess to HRP-C that had previously been incubated with mCBPA was the catalase-like activity of the enzyme.[8–10] This reaction occurs at the level of the compound I·H_2O_2 complex and results in the production of oxygen gas and the direct regeneration of ferric HRP-C. This pathway has been shown to be the main turnover route of HRP-C with H_2O_2.[9,10] When oxygen gas release was

measured using mCPBA-treated HRP-C (15-minute incubation) with 50 mM H_2O_2 as the substrate it was found that the initial level of catalase-like activity (data not shown) was more consistent with the concentration of E_i suggested by simulations (Figure 2B) rather than by measurements of residual peroxidase activity with ABTS (Figure 2A). These data therefore provided further evidence that a proportion of the enzyme was present in the form of high affinity complexes with mCPBA.

In summary, the complexes between HRP-C compounds I or II, and mCPBA have been identified as being functionally equivalent to enzyme that has been inactivated due to the activity of this substrate as a mechanism-based inactivating agent, in as much that the affinity of mCPBA for HRP-C is high enough that the H_2O_2 used in assays with the donor substrate ABTS cannot displace mCPBA unless it is present in very large excess. These observations provide an explanation of the discrepancy between the time dependent falls in activity with ABTS and simulations of the reaction mechanism (Figure 1) performed using accurately determined rate constants from single turnover stopped-flow experiments. The data provide a useful addition to our knowledge of the behaviour of this widely studied enzyme that nevertheless continues to offer further opportunities for research. They also indicate that whilst steady state and transient state kinetic data may produce apparently consistent results it is important to bear in mind other circumstances such as the assay conditions being used.

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