Catalase-like activity of horseradish peroxidase: relationship to enzyme inactivation by H_2O_2

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H_2O_2 is the usual oxidizing substrate of horseradish peroxidase C (HRP-C). In the absence in the reaction medium of a one-electron donor substrate, H_2O_2 is able to act as both oxidizing and reducing substrate. However, under these conditions the enzyme also undergoes a progressive loss of activity. There are several pathways that maintain the activity of the enzyme by recovering the ferric form, one of which is the decomposition of H_2O_2 to molecular oxygen in a similar way to the action of catalase. This production of oxygen has been kinetically characterized with a Clark-type electrode coupled to an oxygraph. HRP-C exhibits a weak catalase-like activity, the initial reaction rate of which is hyperbolically dependent on the H_2O_2 concentration, with values for K_2 (affinity of the first intermediate, compound I, for H_2O_2) and k_3 (apparent rate constant controlling catalase activity) of 4.0 ± 0.6 mM and 1.78 ± 0.12 s^{-1} respectively. Oxygen production by HRP-C is favoured at pH values greater than approx. 6.5; under similar conditions HRP-C is also much less sensitive to inactivation during incubations with H_2O_2. We therefore suggest that this pathway is a major protective mechanism of HRP-C against such inactivation.

Key words: horseradish peroxidase C, oxygen, protection.

INTRODUCTION

Haem peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) catalyses the global reaction in eqn (1) in which an oxidant (ROOH) and a reductant (AH_2) react together:

$$2AH_2 + ROOH \rightarrow 2AH^{\bullet} + ROH + H_2O \quad (1)$$

The reaction proceeds in three distinct steps [1]. First, the native ferric enzyme reacts with the oxidizing substrate (H_2O_2 or another hydroperoxide, ROOH) to yield compound I.

Compound I is not a classical enzyme–substrate complex [2], but rather a reactive intermediate with a higher formal oxidation state (+5 compared with +3 for the resting enzyme). Thus, compound I is capable of oxidizing a range of reducing substrates by a mechanism involving two sequential single-electron steps. The first of those steps leads to the generation of a second enzyme intermediate, compound II (oxidation state +4), which is subsequently reduced to the native enzyme (E) by a second molecule of reducing substrate:

$$E + H_2O_2 \xrightarrow{H_2O} \text{compound I} \xrightarrow{AH_2\ AH^{\bullet}} \text{compound II} \xrightarrow{AH_2\ AH^{\bullet}} E + H_2O \quad (2)$$

In the absence of the usual reducing substrates (AH_2), H_2O_2 can perform a dual role, as oxidant in the formation of compound I and as a typical one-electron donor (reducing) substrate for peroxidase:

$$E + H_2O_2 \xrightarrow{H_2O} \text{compound I} \xrightarrow{H_2O_2\ HO_2^{\bullet}} \text{compound II} \xrightarrow{H_2O_2\ HO_2^{\bullet}} E + H_2O \quad (3)$$

However, eqn (3) does not occur in a straightforward way and a number of additional complexities have been identified: (1) the final step, during which compound II is reduced, is very slow and a new spectroscopically distinguishable species (compound III) appears [3–5]; (2) catalase-like activity, which releases oxygen, has been observed [4,6,7]; and (3) there is a progressive inactivation of the enzyme producing a verdohaemoprotein (P670) devoid of catalytic activity [8–11].

This last phenomenon has for some time been the object of our research: we have focused on it as an example of mechanism-based enzyme inactivation (suicide inactivation). We have tested many of the criteria established for the characterization of this type of inactivation process [12–16], such as partition ratio, time dependence, concentration dependence and saturation kinetics, substrate protection and irreversibility [17–21]. Our extensive kinetic approach has previously allowed us to study the inactivation of a slightly basic horseradish peroxidase (HRP) isoenzyme, HRP-C. As a result, the model shown in Scheme 1 was postulated to describe and summarize the catalytic and inactivation pathways that have been identified [18]. Thus peroxidase in reaction with H_2O_2 will either react via one of the protective pathways (i.e. it will dismutate the peroxide to generate oxygen gas in a catalase-like cycle or will follow the compound III pathway) or it will be inactivated.

To gain a better understanding of the compound III pathway, an exhaustive kinetic study in both the pseudo-steady state and the transient state was performed with the xenobiotic hydroperoxide *m*-chloroperoxybenzoic acid (*m*-CPBA) as the substrate of HRP-C instead of H_2O_2 [22,23].

Because *m*-CPBA cannot complete a catalase-like cycle with HRP-C, it was possible to directly probe the relationship of the compound III and inactivation pathways only. Under these conditions the partition ratio (r) between the compound III route and inactivation decreased to a very low value ($r = 2$) compared with that obtained with H_2O_2 ($r \approx 600$–700). This both confirmed

Abbreviations used: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); AH_2, reducing substrates; HRP, horseradish peroxidase; HRP-C, horseradish peroxidase isoenzyme C; *m*-CPBA, *m*-chloroperoxybenzoic acid; RZ, Reinheitzahl (an indication of the purity of peroxidases given by the ratio A_{403}/A_{275}).

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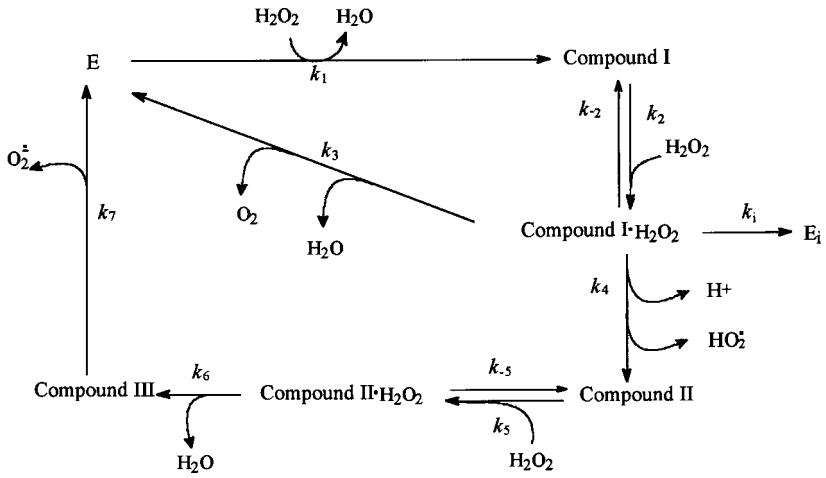

Scheme 1 Model of the reaction of HRP-C with H_2O_2

E and E_i are the native and inactive enzyme, respectively; compound I, II, III are enzyme intermediates; compound I·H_2O_2 and compound II·H_2O_2 are complexes between the respective enzyme intermediates and H_2O_2. Principal features of the model are as follows. (1) The enzyme exhibits two catalytic cycles (catalase-like/peroxidase activities) with distinct rate constants and stoichiometries. (2) The complex compound I·H_2O_2 has a central role. The catalase-like (O_2 releasing) activity involves a direct two-electron reduction of H_2O_2. The peroxidatic (compound III) pathway proceeds stepwise in two single-electron reductions. The inactivation of HRP-C (to give E_i) is the third pathway from the complex. (3) The distribution of enzyme between the two catalytic pathways and one inactivating pathway results from two partition ratios: $r_c = k_3/k_i$ (catalase-like route/inactivation) and $r_{\text{compound III}} = k_4/k_i$ (compound III route/inactivation). The total value of the overall partition ratio (r) is: $r = r_c + r_{\text{compound III}}$ and corresponds to the number of turnovers performed by one mole of enzyme before its inactivation.

the minor contribution of the compound III pathway to the protection of the enzyme and simultaneously suggested that the catalase-like activity of HRP-C was of considerably greater significance.

A transient intermediate (P965) has been shown to be the complex between compound I and hydroperoxide. The identification of P965 supports the validity of the reaction scheme because of the pivotal role played by the [compound I·hydroperoxide] complex as the species from which partition between the various pathways occurs (roles that neither compound II nor compound III performs) [23].

This paper describes the kinetic characterization of the catalase-like activity of HRP-C as measured by O_2 production, both in the initial phase of the reaction and after complete consumption of H_2O_2. The results are linked to the general mechanism of catalysis and inactivation laid out in Scheme 1 and provide additional justification for the model.

EXPERIMENTAL

HRP

Purified horseradish peroxidase (HRP) (type IX; RZ = 3.2) was obtained from Sigma. This preparation was characterized by isoelectric focusing as described previously [20]; the sample was detected as a single band with a pI of 8.5 and confirmed to be isoenzyme C. Its specific activity with the reducing substrate 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was 1322 units/mg.

Chemicals

H_2O_2 (30 %, v/v) and buffer substances (analytical reagent grade) were obtained from Merck. ABTS in the form of the crystallized diammonium salt was supplied by Boehringer Mannheim. All solutions were prepared with deionized water drawn from a Milli-Q system (Millipore).

Oxygen production

Oxygen production was measured with a Clark-type electrode coupled to a Hansatech Oxygraph (Kings Lynn, Norfolk, U.K.). The equipment was calibrated with the tyrosinase/4-t-butylcatechol method [24]. Nitrogen was bubbled through the reaction medium to remove the oxygen. A baseline increase in oxygen concentration of less than 0.5 μM/min without enzyme was obtained. The reaction medium (2 ml) contained H_2O_2 at different concentrations (see the Figures) in the following buffers: 50 mM sodium citrate (pH 3.0, 3.5, 4.5, 5.5 and 5.8), 50 mM sodium phosphate (pH 6.0, 6.3, 6.4, 6.5, 7.0 and 7.5) and 50 mM sodium carbonate (pH 8.0 and 8.5), at 25 °C. The reactions were started by the addition of 10–50 μl of HRP in oxygen-free (nitrogen-bubbled) water. Enzyme concentrations were estimated by measuring the absorbance of solutions at 403 nm, taking ϵ_{403} as 100 $mM^{-1}\cdot cm^{-1}$ [25]. The concentration of H_2O_2 was determined spectrophotometrically, taking ϵ_{240} as 43.6 $M^{-1}\cdot cm^{-1}$ [26]. Two types of experiment were performed with the system described.

Determination of the initial rate of H_2O_2-decomposing activity (V_0)

Values of V_0 were determined at short reaction times in the initial linear phase of oxygen production, from triplicate measurements at each [H_2O_2] and [HRP-C]. The initial linear phase lasted approx. 10–30 s; departure from linearity at later times indicated that enzyme inactivation was being observed, so these later data were not used in calculations. From experiments studying the dependence on [H_2O_2], initial estimates of V_{max} and K_m were obtained by using the Hanes equation, a linear transformation of the Michaelis–Menten equation [27]. These estimates were refined by non-linear regression to a plot of V_0 against [H_2O_2] by using the program SigmaPlot for Windows (version 2; Jandel Scientific Software, San Rafael, CA, U.S.A.).

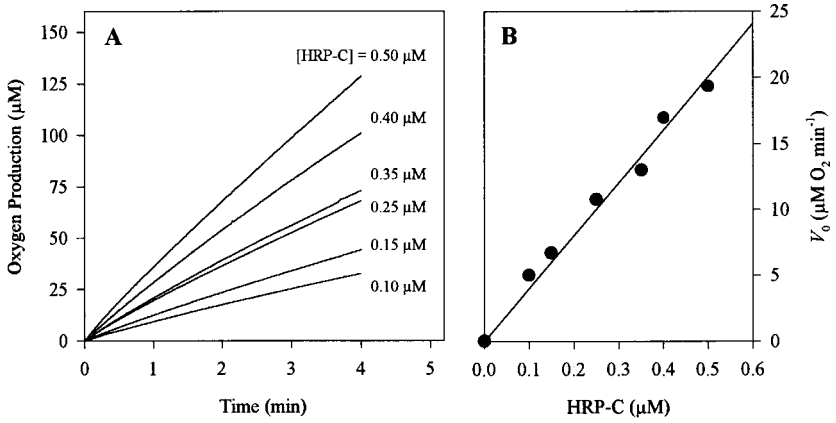

Figure 1 Dependence of oxygen production on time and HRP concentration

(**A**) Time courses of oxygen production in a catalase-like reaction between HRP-C and H_2O_2. The reactions were started by the addition of different concentrations of enzyme, as shown, to a reaction medium containing 5 mM H_2O_2 in 50 mM sodium phosphate buffer, pH 6.5, at 25 °C. (**B**) Plot of initial rate of oxygen production against HRP-C concentration.

Determination of the final oxygen produced at the end of the reaction ($[O_2]_\infty$)

In this case, the end of reaction was reached when no further O_2 production was observed. In all experiments, $[O_2]_\infty$ was less than 0.24 mM (i.e. the oxygen concentration in air-saturated medium at 25 °C).

Peroxidase inactivation and the determination of residual activity

The inactivation of HRP was performed at 25 °C in 1 ml incubations in similar buffers to those used in the oxygraph (pH 3.5–8.5). Each incubation contained enzyme (1 μM) and H_2O_2 at different concentrations (giving $[H_2O_2]$ to [HRP] ratios from 0 to 1500). When the reaction was complete (4 h), aliquots from the incubations were transferred to cuvettes containing an assay mixture comprising 0.5 mM ABTS and 0.2 mM H_2O_2 in 50 mM glycine/HCl buffer, pH 4.5. The peroxidase activity was measured spectrophotometrically by the increase in A_{414} (ϵ 31.1 $mM^{-1}\cdot cm^{-1}$), which is a characteristic of the ABTS oxidation product [28]. The concentration of ABTS was determined spectrophotometrically, taking ϵ_{340} as 36 $mM^{-1}\cdot cm^{-1}$ [28]. The residual enzyme activity (A_r) (expressed as a percentage) was taken as the activity remaining at the end of the reaction (A_t) as a proportion of the initial activity (A_0) [18].

Spectrophotometric assays were recorded on a Perkin-Elmer Lambda-2S UV–visible spectrophotometer interfaced with a PC. The temperature was controlled at 25 ± 0.1 °C with a Haake D1G circulating-water bath with a heater and cooler.

RESULTS AND DISCUSSION

Oxygen production

HRP-C is able to produce molecular oxygen when it is incubated with H_2O_2 as the sole substrate. This oxygen production can be monitored with an oxygraph (Clark electrode). The time-course plots show a short initial linear phase (Figure 1A), permitting the calculation of the initial reaction rates of oxygen production (V_0) before enzyme inactivation by H_2O_2 becomes significant. The V_0 values exhibited a linear dependence on enzyme concentration across the range 0–0.5 μM (Figure 1B). In addition, the hyperbolic dependence of V_0 on the H_2O_2 concentration revealed that saturation kinetics were being observed under steady-state conditions; thus values for K_m and V_{max} could be obtained from the data (Figure 2).

The present results provide evidence for a weak but measurable catalase-like activity in HRP-C, of the same order of magnitude as that reported by Nakajima and Yamazaki [4] for a similar reaction observed when they studied the mechanism of oxyperoxidase formation.

Thus HRP-C has a low affinity for H_2O_2 (K_m is in the millimolar range), similar to that of catalases [29] and chloroperoxidases [30]. However, HRP-C exhibits saturation. This is an important divergence from the behaviour of the typical catalases, which either do not show saturation or do so only under severe and non-physiologically relevant conditions (up to 5 M H_2O_2) [31]. HRP-C is closer in behaviour to chloroperoxidase, which exhibits saturation kinetics at similar H_2O_2 concentrations [32].

The efficiency of HRP-C ($k_{cat}/K_m \approx 10^2$–10^3 $M^{-1}\cdot s^{-1}$) is close to the bottom of a ranking in which the catalases [29] and bacterial catalase–peroxidases [33–35] ($k_{cat}/K_m \approx 10^6$ $M^{-1}\cdot s^{-1}$) occupy the highest positions, followed by chloroperoxidases ($k_{cat}/K_m \approx 10^4$ $M^{-1}\cdot s^{-1}$) [32]. The activity of HRP-C is low but can be distinguished from that of ascorbate peroxidase, which has no observable catalase activity [36].

The pH profile for the catalase-like reaction of HRP-C is shown in Figure 3. The maximum rate of O_2 production is reached in a plateau over the pH range 6.5–8.5, indicating that acid media are unfavourable for the catalase-like cycle. The strong pH dependence of HRP-C's catalase-like activity, like its saturation (above), is different from catalase, whose activity is essentially pH independent in the pH range 4.7–10.5 [37].

It is perhaps unsurprising that HRP-C and catalase exhibit divergent behaviours even though the overall reaction catalysed is the same (i.e. the dismutation of H_2O_2 to H_2O and O_2 gas): the haem peroxidases and catalases are unrelated and structurally distinct enzyme families [38–40]. Catalases tend to be homotetramers that bind one haem group per subunit, the tetrameric structure being required for their activity [41]. The proximal ligand to the ferric iron centre is a tyrosine; catalytically essential histidine and asparagine residues have been identified at the active site [40,41]. The reaction mechanism under true turnover conditions with H_2O_2 is still open to discussion but it is clear that compound I is involved in catalysis, whereas compound II is not

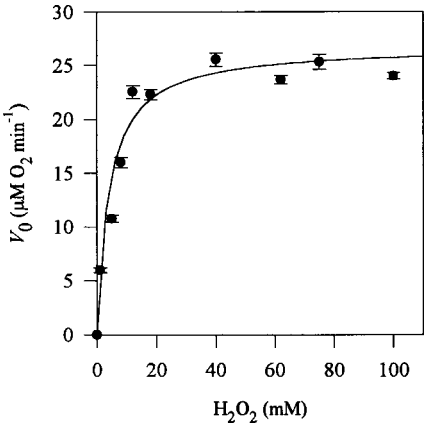

Figure 2 Hyperbolic H_2O_2 concentration dependence of the initial rate of oxygen production

The reactions were started by the addition of enzyme (0.25 μM) to media containing different amounts of H_2O_2 in 50 mM sodium phosphate buffer, pH 6.5, at 25 °C.

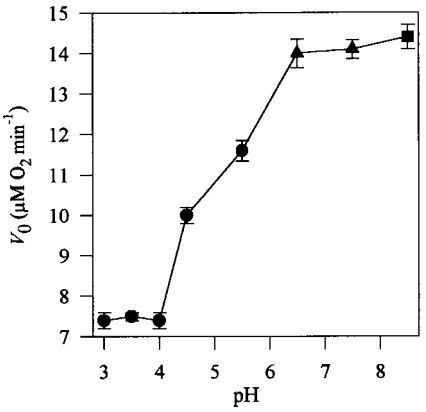

Figure 3 pH-dependence of the initial rate of oxygen production

The reactions were started by the addition of enzyme (0.25 μM) to reaction media containing H_2O_2 (10 mM) in 50 mM buffer (●, sodium citrate, pH 3.0–5.5; ▲, sodium phosphate, pH 6.5–7.5; ■, sodium carbonate, pH 8.5).

[37]. Catalase compound I has been isolated by using non-oxidizable substrates (e.g. peracetic acid) [42], and contains an oxyferryl iron centre and a porphyrin π-cation radical, as does compound I of HRP-C [43]. The classical plant peroxidases, such as HRP-C, are generally monomeric with a single haem group [38] (although other related peroxidases might be dimers, for example ascorbate peroxidase [44], or interestingly, tetramers, such as bacterial catalase–peroxidases [45,46]). The haem ferric iron's proximal ligand is histidine; conserved arginine and histidine residues are present at the active site [39, 47]. The typical peroxidatic reaction (see the Introduction section) involves the formation of compounds I and II but the catalase-like cycle of HRP-C involves only compound I (see Scheme 1). It therefore seems likely that the mechanisms of HRP-C (acting as a catalase) and catalase are basically similar but that at the molecular level a range of factors exist that account for the observed differences in activity.

Steady-state kinetic approach

The reaction of HRP-C with H_2O_2 in the absence of other substrates as shown in Scheme 1 will evolve in the transition state towards total inactivation of the enzyme. However, the rate constants controlling the catalase-like cycle are overwhelmingly large compared with those controlling the other pathways; thus at short reaction times a very good approximation to the steady state is observed from measurements of O_2 production.

The results described in Figures 1 and 2 are consistent with the reaction model shown in Scheme 1 and can be interpreted with it. Catalase activity implies a pathway that does not pass through compound II. The native enzyme (E) reacts with H_2O_2 to form compound I, which then forms a complex with a second H_2O_2 molecule. The formation of the compound I·H_2O_2 complex is important as an explanation of the saturation effect at higher H_2O_2 concentrations (Figure 2). At short reaction times a kinetic analysis can be developed by following an approach similar to that used by Sun et al. [32] to interpret the catalase activity of chloroperoxidase.

The equations in the steady state are:

$$[E]_T = [E] + [\text{compound I}] + [\text{compound I} \cdot H_2O_2] \quad (4)$$

where $[E]_T$ is the total amount of enzyme in the system and [E] is the concentration of native enzyme,

$$\frac{d[O_2]}{dt} = k_3[\text{compound I} \cdot H_2O_2] \quad (5)$$

and

$$K_2 = \frac{[\text{compound I}]\,[H_2O_2]}{[\text{compound I} \cdot H_2O_2]} \quad (6)$$

The following equation can be formulated in the steady state:

$$\frac{d[O_2]}{dt} = \frac{k_3[E]_T[H_2O_2]}{K_2 + [H_2O_2]} \quad (7)$$

The results presented in Figure 2 can be fitted to eqn (7) for rectangular hyperbolae; thus the values for K_3 and k_2 are obtained. Since $V_{max} = k_3[E_T]$, $k_3 = 1.78 \pm 0.12\ s^{-1}$ and $K_m = K_2 = 4.0 \pm 0.6$ mM.

It is notable that the values obtained for the two constants (k_3 and K_2) characterizing catalase activity are similar to those obtained when we studied the time-dependent inactivation of the enzyme [18]. The results presented here by a different approach (oxygen production) give added support to the validity of Scheme 1 as a model of the action of HRP-C.

Enzyme inactivation

The catalase-like oxygen-producing reaction between HRP-C and H_2O_2 just described is accompanied by a progressive loss of enzyme activity. This process exhibits biphasic kinetics and has previously been shown [18] to be due to mechanism-based (suicide) inactivation. In this case, a partition ratio (r), defined as the number of turnovers that the enzyme undergoes before its total inactivation, can be calculated as a useful parameter characterizing the process. To obtain r, samples of enzyme are incubated with a range of concentrations of H_2O_2 and the residual activity is measured when the endpoint of the incubation has been reached (i.e. when all the H_2O_2 has been consumed or

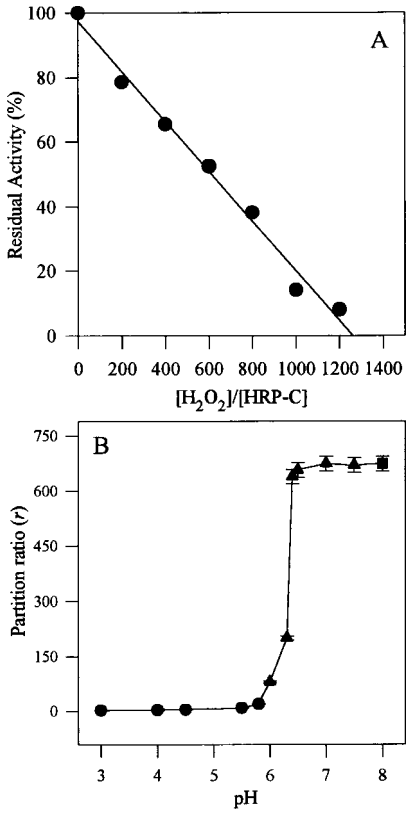

Figure 4 Sensitivity of HRP to inactivation at different molar ratios of H_2O_2 and pH values

(**A**) Plot of residual activity against the $[H_2O_2]$-to-[HRP-C] ratio. Residual activity was measured with ABTS as substrate after incubations lasting for 4 h. The line was fitted and the value of the intercept at the *x*-axis was used to calculate *r* (see the text). (**B**) Variation in *r* with pH (50 mM buffers: ●, sodium citrate, pH 3.0–5.8; ▲, sodium phosphate, pH 6.0–7.5; ■, sodium carbonate, pH 8.0).

all the enzyme inactivated). In Figure 4(A) the residual activity (A_r) has been plotted against the ratio $[H_2O_2]/[HRP\text{-}C]$. The observed value of the intercept on the abscissa of the straight-line fit to the data indicates the number of equivalents of H_2O_2 needed to eliminate HRP-C activity totally and can be used to calculate *r*.

The values of *r* for various HRP isoenzymes and mutants have previously been calculated by using a simple model of inactivation [20,21]. However, with the use of the complete mechanism shown in Scheme 1 a more rigorous treatment of the calculation of *r* can be performed. In the Appendix the derivation of the equation relating the loss of enzyme activity to $[H_2O_2]/[HRP\text{-}C]$ is shown, yielding:

$$A_r = 1 - \frac{1}{2r+2}\,\frac{[H_2O_2]}{[HRP\text{-}C]} \qquad (8)$$

From Figure 4(A), $A_r = 0$ when $[H_2O_2]/[HRP\text{-}C] = 1250 \pm 80$ at pH 6.5. Introducing this value into eqn (8), the value of the partition ratio, $r = 624 \pm 40$, is obtained. This result is in good agreement with the independent values of *r* determined from kinetics and by the approximation method [18–21].

The values of *r* obtained from incubation experiments were strongly dependent on pH, as shown in Figure 4(B). Under more acidic conditions the enzyme was found to be very much more sensitive to inactivation than at neutral pH. The inflexion point of the pH-dependence curve was at pH 6.4. These data broadly parallel the pH dependence of the catalase-like activity (Figure 3) and suggest that this pathway is a major protective mechanism for HRP-C against inactivation by H_2O_2.

Table 1 Kinetic constants and partition ratios obtained at pH 6.5 from direct measurements of O_2 production and the inactivation of HRP-C by H_2O_2

The constants are: k_3, apparent rate constant of the catalase-like pathway; k_i, rate constant of inactivation; K_2, dissociation constant of the compound $I \cdot H_2O_2$ complex; *r*, partition ratio (number of catalytic cycles given by the enzyme before its inactivation). Results are means ± S.E.M.

Constant	Value
k_3 (s^{-1})	1.78 ± 0.12
K_2 (mM)	4.0 ± 0.6
r	700 ± 100*
	624 ± 40†
10^3 k_i (s^{-1})	2.5 ± 0.2

* Partition ratio from oxygen measurements; see Appendix, eqn (A2).
† Partition ratio from end-point incubations; see Appendix, eqn (A9).

A further independent estimate of *r* can be made by following the total generation of O_2 during the reaction. Over the [HRP-C] range 30–200 nM the quantity of product accumulated at the end of the reaction, the so-called infinite product ($[O_2]_\infty$) when the enzyme has been inactivated was obtained. A plot of $[O_2]_\infty$ against [HRP-C] gave a linear relationship, passing through the origin, with a slope representing the partition ratio ($r = [O_2]_\infty/[HRP\text{-}C] = 700 \pm 100$) for the oxygen-producing activity exhibited by the enzyme.

Thus consistent values of *r* can be obtained by using different experimental procedures and, although the data supplied by these methods are only approximate, they agree with the value obtained from the kinetic study made previously on the inactivation of HRP-C by H_2O_2 [18].

It is known that the compound III pathway is of relatively minor importance, the number of turnovers that occur via this route being very much smaller than the number for the catalase-like cycle [18,22,23]. Therefore, since $r_c \gg r_{\text{compound III}}$ and from the expressions defined in the Appendix, it can be seen that:

$$r \approx r_c = k_3/k_i \qquad (9)$$

This new expression for *r* [eqn (9)] is useful because k_3 was obtained from the study of oxygen production (Figure 2) and thus it is now possible to calculate $k_i = (2.5 \pm 0.2) \times 10^{-3}$ s^{-1}.

Conclusions

Table 1 gives the kinetic constants and parameters that characterize the inactivation of HRP-C by H_2O_2 by using measurements of O_2 production and the suicide inactivation of the enzyme. Some interesting observations can be made about the data in Table 1, which illustrate the similarity of the results obtained with the different experimental methods: loss of enzyme activity with time [18], endpoint incubations [20,21], inactivation by another hydroperoxide (*m*-CPBA) [22,23] and oxygen production (the present paper). Nakajima and Yamazaki [4] found that approx. 500 equiv. of H_2O_2 were required to form HRP-C compound III. Thus a considerable quantity of H_2O_2 must be dissipated to generate compound III. This confirms the dominant role that we have assigned in our mechanism to the catalase-like

pathway of peroxidase compared with the compound III pathway: although both processes protect the enzyme against inactivation by H_2O_2, the catalase pathway is much more important.

The three reactions, namely (1) formation of compound III, (2) a catalase-like reaction and (3) enzyme inactivation by H_2O_2, have also been described in chloroperoxidase [32], lactoperoxidase [4,48,49] and lignin peroxidase [50,51]. With lignin peroxidase approx. 20 equiv. of H_2O_2 were needed to generate compound III [50], which is rather lower than for HRP-C. However, these results were obtained at pH 3.0, in contrast with pH 8.9 with HRP-C [4]. We found (Figure 3) that the catalase-like cycle of HRP-C was pH-dependent and greatly disfavoured under acidic conditions, in which the enzyme turns over via the compound III pathway and is also much more sensitive to inactivation as observed from the pH-dependence of the partition ratios in incubation experiments (Figure 4B). Lignin peroxidase can exhibit a similar pH profile and it would be very interesting to compare the data for the two enzymes to see whether the quantitative difference in the number of equivalents of H_2O_2 required to form compound III is due purely to pH. The pH dependence data for HRP-C suggest that an ionizable group with a pK_a of approx. 6.4 (possibly a histidine residue) might be implicated at the mechanistic level in the active site, in agreement with other studies [4]. Mutant HRP-Cs, such as R38K and H42L, in which active-site residues have been changed, exhibit altered activity and are more sensitive to inactivation [21,52]. It is evident that the pH of the medium is important in those industrial situations in which peroxidase is exposed to high H_2O_2 levels (e.g. bioreactors and detergents) and in which enzyme inactivation should be minimized.

Physiologically, the catalase-like activity of peroxidase might seem largely irrelevant because of the presence of reducing substrates that normally take part in peroxidatic activity and protect the enzyme from inactivation [17,53]. However, under conditions of oxidative stress or during the protective H_2O_2 burst of plants in response to pathogenic attack, an elevated level of oxidants compared with reductants can occur. In such situations the various isoperoxidases present in a cell are more susceptible to inactivation and the catalase-like reaction will act to protect them to a certain extent, although the level of protection will be different for each peroxidase depending on their susceptibility and subcellular localization. The apoplastic and vacuolar peroxidases are exposed to larger fluctuations in peroxide concentration than those in the cytoplasm. Cytoplasmic ascorbate peroxidase does not possess observable catalase-like activity and is very sensitive to inactivation [36,54]. However, an apoplastic peroxidase from *Lupinus albus* hypocotyls [55] shows behaviour very similar to that described here for HRP-C.

In summary, we have characterized a weak catalase activity exhibited by HRP-C when it is incubated with H_2O_2 as the sole substrate; this activity is a major protective mechanism for the enzyme against inactivation. Comparable catalase-like activity is probably present in other secretory peroxidases and the activity has implications for the function of peroxidases *in vivo* and in industrial applications.

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APPENDIX

Determination of the partition ratio, *r*

A relationship between the residual activity of the enzyme, the partition ratio and the substrate-to-enzyme ratio can be obtained in accordance with Scheme 1. The stoichiometries of the three proposed routes are (1) the catalase-like pathway:

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

(2) the compound III pathway:

$$3H_2O_2 \rightarrow H^+ + HO_2^{\bullet} + O_2^{\bullet -} + 2H_2O$$

but the instability of anion superoxide can lead to its dismutation and then:

$$3H_2O_2 \rightarrow O_2 + H_2O_2 + 2H_2O$$

which can be simplified to the same reaction as in the catalase-like route:

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

and (3) the inactivation pathway:

$$2H_2O_2 \rightarrow H_2O + E_i$$

Therefore the balance for the substrate is:

$$[H_2O_2]_{total} = 2[O_2]_c + 2[O_2]_{compound\ III} + 2[E_i] \quad (A1)$$

where $[O_2]_c$ and $[O_2]_{compound\ III}$ are the concentrations of oxygen produced in pathways (1) and (2) respectively, and $[E_i]$ is the concentration of inactivated enzyme.

In addition, because:

$$r_c = \frac{[O_2]_c}{[E_i]} \quad (A2)$$

and:

$$r_{compound\ III} = \frac{[O_2]_{compound\ III}}{[E_i]} \quad (A3)$$

where r_c and $r_{compound\ III}$ are the numbers of turnovers of the enzyme through the catalase-like pathway and the compound III pathway respectively, then:

$$[H_2O_2]_{total} = 2r_c[E_i] + 2r_{compound\ III}[E_i] + 2[E_i] \quad (A4)$$

$$[E_i] = \frac{[H_2O_2]_{total}}{2r_c + 2r_{compound\ III} + 2} \quad (A5)$$

The concentration of active enzyme remaining at the end of the reaction, $[E_a]$, is simply the difference between the initial enzyme concentration $[E_0]$ and the concentration of inactivated enzyme $[E_i]$:

$$[E_a] = [E_0] - [E_i] \quad (A6)$$

By introducing eqn (A5) into eqn (A6):

$$[E_a] = [E_0] - \frac{[H_2O_2]_{total}}{2r_c + 2r_{compound\ III} + 2} \quad (A7)$$

and by dividing by $[E_0]$, the residual activity (A_r) is given:

$$A_r = \frac{[E_a]}{[E_0]} = 1 - \frac{1}{2r_c + 2r_{compound\ III} + 2} \frac{[H_2O_2]_{total}}{[E_0]} \quad (A8)$$

Because $r = r_c + r_{compound\ III}$ the final expression is:

$$A_r = 1 - \frac{1}{2r + 2} \frac{[H_2O_2]_{total}}{[E_0]} \quad (A9)$$

Thus, by plotting A_r against $[H_2O_2]_{total}/[E_0]$ and using eqn (A9), it is possible to calculate the partition ratio, r, for this particular mechanism (Scheme 1).

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