

## A comparative study of the inactivation of wild-type, recombinant and two mutant horseradish peroxidase isoenzymes C by hydrogen peroxide and *m*-chloroperoxybenzoic acid

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The mechanism-based inactivation of four horseradish peroxidase (HRP-C) enzyme variants has been studied kinetically with either hydrogen peroxide or the xenobiotic *m*-chloroperoxybenzoic acid (*m*ClO<sub>2</sub>-BzOH) as sole substrate. The concentration and time dependence of inactivation was investigated for the wild-type plant enzyme (HRP-C), the unglycosylated recombinant enzyme (HRP-C\*), and two site-directed mutants with Phe143 replaced by Ala ([F143A]HRP-C\*) or Arg38 replaced by Lys ([R38K]HRP-C\*). The number of turnovers (*r*) of H<sub>2</sub>O<sub>2</sub> required to completely inactivate the enzymes was found to vary between the different enzymes with HRP-C being most resistant to inactivation (*r* = 625), HRP-C\* and [F143A]HRP-C\* being approximately twice as sensitive (*r* = 335 and 385, respectively) in comparison, and [R38K]HRP-C\* being inactivated much more easily (*r* = 20). In the cases of HRP-C\* and [F143A]HRP-C\*, compared to HRP-C the differences were due to the absence of glycosylation on the exterior of the proteins, whilst the [R38K]HRP-C\* variant exhibited a distinct mechanistic difference. When *m*ClO<sub>2</sub>BzOH was used as the substrate the differences in sensitivity to inactivation disappeared. The values of *r* were all around 3 reflecting the strong affinity of *m*ClO<sub>2</sub>BzOH for the active site. The apparent rate constant for inactivation by H<sub>2</sub>O<sub>2</sub> was found to be about twofold higher in [R38K]HRP-C\* than the other enzymes and the catalytic constant for turnover of H<sub>2</sub>O<sub>2</sub> was approximately ten times lower. The affinity of compound I for H<sub>2</sub>O<sub>2</sub> leading to the formation of a transitory intermediate implicated in the inactivation of peroxidase decreased in the order HRP-C, HRP-C\*, [F143A]HRP-C\*, [R38K]HRP-C\*.

**Keywords:** horseradish peroxidase; recombinant; mutant; hydrogen peroxide; suicide inactivation.

Horseradish peroxidase (HRP) (donor:hydrogen peroxide oxidoreductase) is a classical haem peroxidase and a member of the superfamily of peroxidase enzymes found in plants, fungi and bacteria. Following the classification of Welinder [1], HRP is a class III secretory enzyme which is involved in the formation of free radicals vital in polymerisation reactions such as lignification and suberization. It occurs in a wide variety of isoforms [2], slightly basic (cationic) HRP-C being the most abundant [3].

The enzyme has been characterised as a single polypeptide chain, heavily glycosylated (18% by mass) [4] and containing a single high-spin ferric protohaem IX prosthetic group; two

calcium ions are bound [5, 6], one of which is important for protein folding [7, 8]. The detailed X-ray crystallographic structure has not yet been elucidated for any of the isoenzymes of HRP although preliminary co-ordinates are available for the highly basic isoenzyme, E5 [9]. Sequence comparison with yeast cytochrome *c* peroxidase (CCP), of which the high-resolution X-ray structure is known [10], has been used to produce a model of the enzyme [11] and, in particular, its active site containing the haem with His170 at the fifth (proximal) iron co-ordination position, and conserved His42 and Arg38 residues important for peroxide binding and reaction on the distal side. One haem edge ( $\delta$ -*meso* carbon) is thought to be exposed to solvent and to constitute part of the binding site for some reductant substrates [12].

The catalytic cycle of HRP proceeds via a number of distinct steps. In the first step, peroxide (normally H<sub>2</sub>O<sub>2</sub>) binds and reacts at the active site to generate compound I by a two-electron process [13] in which the haem iron is oxidised to the oxyferryl (Fe<sup>IV</sup>=O) state, with the second electron removed from the porphyrin ring to generate a  $\pi$ -cation radical [4] where the charge is delocalised over the ring rather than transferred to an amino acid as is observed in yeast CCP [14]. The second product of the reaction is water. The apparent second-order rate constant for the formation of compound I,  $k_1 = 1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  [4]. In the presence of a suitable reductant substrate the second step of the cycle involves in most cases a single electron oxidation of

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**Abbreviations.** HRP-C, horseradish peroxidase isoenzyme C (plant enzyme); HRP-C\*, recombinant horseradish peroxidase isoenzyme C; [F143A]HRP-C\*, recombinant horseradish peroxidase isoenzyme C in which Phe143 has been replaced by Ala; [R38K]HRP-C\*, recombinant horseradish peroxidase isoenzyme C in which Arg38 has been replaced by Lys; *m*ClO<sub>2</sub>BzOH, *m*-chloroperoxybenzoic acid; *m*ClBzOH, *m*-chlorobenzoic acid; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); CCP, cytochrome *c* peroxidase.

**Enzymes.** Horseradish peroxidase, acovado peroxidase (EC 1.11.1.7); cytochrome-*c* peroxidase (EC 1.11.1.5).

the substrate by compound I in which the  $\pi$ -cation radical is discharged and oxyferryl compound II and a free radical product are formed. The rate of this reaction is dependent on the substrate [15] but is lower than the previous process. The third step results in the oxidation of a second substrate molecule by reduction of compound II back to the ferric enzyme. Again the rate of reaction is lower (10–20-fold), compound II being observed under steady-state turnover conditions [16]. A limited number of substrates appear to undergo one double electron oxidation by compound I with the concomitant direct regeneration of ferric enzyme, these include halides (iodide) and thioanisoles [17–20]. Furthermore, in the case of thioanisole sulfoxidation, the oxygen is derived from the oxyferryl iron group indicating that HRP is acting overall as a monooxygenase [20].

In the absence of the usual reductant substrates,  $\text{H}_2\text{O}_2$  will react with compound I as an electron donor (reductant) [21, 22]. Reduction of compound I may proceed either via a catalase-like two-electron process, resulting in the formation of molecular oxygen, or in two single-electron transfers in which compound II, compound III and superoxide radical anion ( $\text{O}_2^-$ ) are formed [23]. However, the reaction of HRP with peroxide only also leads to the progressive inactivation of the enzyme. Inactivation results in the generation of a number of inactive chromophores the final product being a verdohaemoprotein, P-670 [24]. The catalytic and compound III (superoxide anion) generating activities of HRP have a protective effect against inactivation and the partition between protection and inactivation probably occurs at the [compound I  $\cdot$   $\text{H}_2\text{O}_2$ ] complex [24, 25]. The catalase-like process is the major protective mechanism [25]. This is supported by the replacement of  $\text{H}_2\text{O}_2$  as substrate by the xenobiotic pseudo-substrate *m*-chloroperoxybenzoic acid ( $m\text{ClO}_2\text{BzOH}$ ) which cannot undergo a catalytic dismutation and inactivates HRP-C approximately 100 times more effectively than  $\text{H}_2\text{O}_2$  [26].

In order to examine some of the structural requirements for the processes of protection and inactivation, we have studied the reactions of  $\text{H}_2\text{O}_2$  and  $m\text{ClO}_2\text{BzOH}$  with recombinant enzyme (HRP-C\*) [27] produced by refolding after expression in *Escherichia coli* and two mutant enzymes ([R38K]HRP-C\* and [F143A]HRP-C\*) produced by site-directed mutagenesis [28].

HRP-C\* is identical to the wild-type plant enzyme in amino acid sequence, except for an additional amino-terminal Met, but is non-glycosylated, thus it provides a way of examining the role of the sugars attached to the wild-type enzyme.

In the [R38K]HRP-C\* mutant the conserved Arg38 residue in the active site has been changed to a Lys. Poulos and Kraut [29] proposed a general acid/base catalytic mechanism for hydrogen peroxide binding and reduction in yeast CCP with essential roles for two highly conserved residues in the distal heme pocket, Arg48 and His52, which are equivalent to Arg38 and His42 in HRP. The CCP variants [R48K]CCP and [R48L]CCP, react with hydrogen peroxide 2-fold and 200-fold slower, respectively, than does native CCP [30, 31]. It was concluded that a basic residue at position 48 in CCP is not an obligate requirement for the formation of an oxyferryl intermediate and that the distal His plays a far more significant role [30]. The ligation state and steady-state activity of [R38K]HRP-C\* have already been investigated and show a pH-dependence dramatically altered from those of HRP-C\* or the plant enzyme [32]. In contrast to the corresponding CCP mutant, a pre-steady-state kinetic analysis of [R38K]HRP-C\* has shown a 500-fold decrease in the rate of compound I formation which involves a reversibly formed intermediate [33]. Further more, compound II could not be observed kinetically, suggesting that in contrast to the wild-type peroxidase, formation of resting enzyme from compound II was faster than the formation of compound II from compound

I. In this paper we report on the implications for the inactivation of [R38K]HRP-C\* by peroxide of these kinetic changes, in the light of an existing scheme for mechanism-based inactivation by peroxide [25].

[F143A]HRP-C\* is a hyperactive (with the reductant substrate ABTS) peroxidase mutant in which Phe143 has been replaced by a smaller Ala residue. Phe143 is believed to be situated on the periphery of the substrate access channel and was initially tentatively identified from NMR studies as one of a pair of highly mobile Phe residues (normally labelled PheA and PheB) [34, 35] potentially implicated in the binding of aromatic substrates close to the  $\delta$ -meso carbon of the haem. Subsequent NMR characterization of the same [F143A]HRP-C\* mutant studied in this work has shown that it is neither PheA nor PheB [36]. We have studied [F143A]HRP-C\* in order to investigate the effect on inactivation by peroxide of a mutation to a residue not directly involved in peroxide binding.

## MATERIALS AND METHODS

**Enzymes.** Purified horseradish peroxidase (HRP) was obtained from Sigma Chemical Co., type IX, purity index ( $A_{403\text{ nm}}/A_{275\text{ nm}}$ ) = 3.15. The specific activity ( $1322\text{ }\mu\text{mol mg}^{-1}\text{ min}^{-1}$ ) was estimated using 0.2 mM  $\text{H}_2\text{O}_2$  and 0.5 mM ABTS as reductant substrate in 50 mM Gly/HCl pH 4.5 at 25°C. The enzyme sample was characterized by isoelectric focussing using the method presented below. The commercial sample exhibited only one major band using both protein-silver stain and peroxidase-specific stain; this band possessed an isoelectric point (pI) of 8.5. A minor band (less than 10%) was seen with pI = 6.8. The enzyme concentrations of HRP type IX were estimated by measuring the absorbance of solutions at 403 nm using  $\epsilon_{403\text{ nm}} = 100\text{ mM}^{-1}\text{ cm}^{-1}$  [37].

The recombinant, HRP-C\*, was expressed in *E. coli* from a synthetic gene; the inactive polypeptide, containing only traces of haem, was extracted from inclusion bodies and folded in the presence of haem and  $\text{Ca}^{2+}$ . After chromatographic purification following previously determined procedures, active non-glycosylated enzyme was obtained [27]. The mutant enzymes, [F143A]HRP-C\* and [R38K]HRP-C\*, were generated by site-directed mutagenesis of the synthetic gene, expressed and purified in the same way [28]. The expected sequence changes in the mutants were confirmed by DNA-sequence analysis. The purity index and the enzymic activity with ABTS for each synthetic HRP were: HRP-C\*, 2.34 and  $1271\text{ }\mu\text{mol mg}^{-1}\text{ min}^{-1}$ ; [F143A]HRP-C\*, 2.82 and  $1779\text{ }\mu\text{mol mg}^{-1}\text{ min}^{-1}$ ; [R38K]HRP-C\*, 2.30 and  $39\text{ }\mu\text{mol mg}^{-1}\text{ min}^{-1}$ . All these synthetic peroxidases were characterized by isoelectric focussing. When peroxidase-specific stain was used, only two bands appeared in each case, with pI values of 8.7 (which represented about 90% of the peroxidase present) and 7.5. The enzyme concentrations were estimated using the following molar absorption coefficients: HRP-C\*,  $\epsilon_{403\text{ nm}} = 100\text{ mM}^{-1}\text{ cm}^{-1}$ ; [F143A]HRP-C\*,  $\epsilon_{403\text{ nm}} = 124\text{ mM}^{-1}\text{ cm}^{-1}$  (Smith, A. T., unpublished); [R38K]HRP-C\*,  $\epsilon_{405\text{ nm}} = 146\text{ mM}^{-1}\text{ cm}^{-1}$  [38].

**Chemicals.** *m*-Chloroperoxybenzoic acid ( $m\text{ClO}_2\text{BzOH}$ ) in the solid state was supplied by Aldrich Chemical Co., containing *m*-chlorobenzoic acid ( $m\text{ClBzOH}$ ) as an impurity. One sample of  $m\text{ClO}_2\text{BzOH}$  was used which, according to the supplier, had a purity between 80–90% (lot 3800511). The actual purity was estimated by  $^1\text{H}$ -NMR using a Varian Unity 300-MHz spectrometer. The samples (50 mg) were dissolved in ( $^2\text{H}_2$ )chloroform; tetramethylsilane was used as a signal reference. The peak signals corresponding to  $m\text{ClBzOH}$  and  $m\text{ClO}_2\text{BzOH}$  were identified using  $m\text{ClBzOH}$  (99% purity) as standard and was in ac-

cordance with the Aldrich Library of NMR spectra [39]. Using this protocol, the actual amount of  $m\text{ClO}_2\text{BzOH}$  in the sample was determined at 75%.

However, for the inactivation assays, the  $m\text{ClO}_2\text{BzOH}$  samples were purified by recrystallization from light petroleum ether (b.p. 40–60°C)/diethylether (3:1, by vol.) in accordance with Davies et al. [40]. After purification, the product obtained was more than 96% pure (NMR analysis and a single melting point at 92–94°C, corresponding to  $m\text{ClO}_2\text{BzOH}$ ).

Solutions of  $m\text{ClO}_2\text{BzOH}$  were made up in absolute ethanol/water (1:1, by vol.) immediately prior to use. The concentration was estimated using a calculated  $\epsilon_{232\text{ nm}} = 8940\text{ M}^{-1}\text{ cm}^{-1}$ , the value being corrected by the purity factor obtained from NMR.

In some experiments, samples of  $m\text{ClO}_2\text{BzOH}$  without further purification were used. Solutions prepared from this material were corrected by the NMR purity factor, and assayed to measure the rate of HRP inactivation. Results similar to those obtained with purified  $m\text{ClO}_2\text{BzOH}$  were obtained in every case. Moreover, the addition of pure  $m\text{ClBzOH}$  to a medium containing HRP did not produce any changes in the enzyme spectrum and, also, the addition of  $m\text{ClBzOH}$  did not affect the ABTS oxidation catalyzed by HRP. Therefore,  $m\text{ClBzOH}$  did not seem to be an alternative substrate of HRP and so had no effect on the reaction studied.

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), in the form of the crystallized diammonium salt, was supplied by Boehringer-Mannheim. The concentration of ABTS was determined using  $\epsilon_{340\text{ nm}} = 36\text{ mM}^{-1}\text{ cm}^{-1}$  [41]. Hydrogen peroxide (30%, by vol.) was from Merck. The concentration of  $\text{H}_2\text{O}_2$  was determined by measuring absorbance at 240 nm using  $\epsilon_{240\text{ nm}} = 43.6\text{ M}^{-1}\text{ cm}^{-1}$  [42]. Buffer substances (analytical reagent grade) were also obtained from Merck. All solutions were prepared using deionized water obtained from a Milli-Q system (Millipore).

**Equipment.** Spectrophotometric measurements were recorded using a Perkin-Elmer Lambda-2 UV-VIS spectrophotometer interfaced on-line with an Olivetti PCS/386. SX computer. The temperature was thermostatically controlled at 25°C using a Haake D1G circulating water bath with a heater/cooler to a precision of  $\pm 0.1^\circ\text{C}$ .

Isoelectric focussing was done on a PhastSystem (Pharmacia-LKB) using the PhastImage gel analyzer and the Phast-Image software v. 1.0 (Pharmacia-LKB).

**Inactivation of peroxidase.** HRP was inactivated at 25°C in 0.5-ml incubations of 50 mM sodium phosphate pH 6.5 containing a fixed amount of the enzyme (0.5  $\mu\text{M}$ ). The reactions were started by the addition of  $\text{H}_2\text{O}_2$  (over a range of concentrations). At specified time intervals, 10- $\mu\text{l}$  aliquots of the incubation mixtures were transferred to cuvettes containing 2 ml of an assay mixture composed of 0.5 mM ABTS and 0.2 mM  $\text{H}_2\text{O}_2$  in 50 mM Gly/HCl pH 4.5. The peroxidase activity was measured by the increase in absorbance at 414 nm, which is characteristic for the ABTS oxidation product ( $\epsilon_{414\text{ nm}} = 31100\text{ M}^{-1}\text{ cm}^{-1}$ ) [41]; neither ABTS nor HRP present in the assay mixture interfered with the measurement. A minimum of four incubation assays for each peroxide concentration were made. The residual enzymic activity ( $A_r$ ) was taken as the enzymic activity remaining ( $A_t$ ) as a percentage of the initial activity ( $A_0$ ). The data plot and the non-linear fittings were made using SigmaPlot for Windows (Jandel Scientific Software, 2591, Kerner Blvd., San Rafael, CA 94901, USA).

The dependence of inactivation on the molar concentration of  $m\text{ClO}_2\text{BzOH}$  or  $\text{H}_2\text{O}_2$  was measured in 0.2-ml incubations containing the enzyme (0.25  $\mu\text{M}$ ) and peroxide at the required ratio in 50 mM sodium phosphate pH 6.5. The residual activity with ABTS was assayed after 1 h ( $m\text{ClO}_2\text{BzOH}$ ) or 4 h ( $\text{H}_2\text{O}_2$ ).

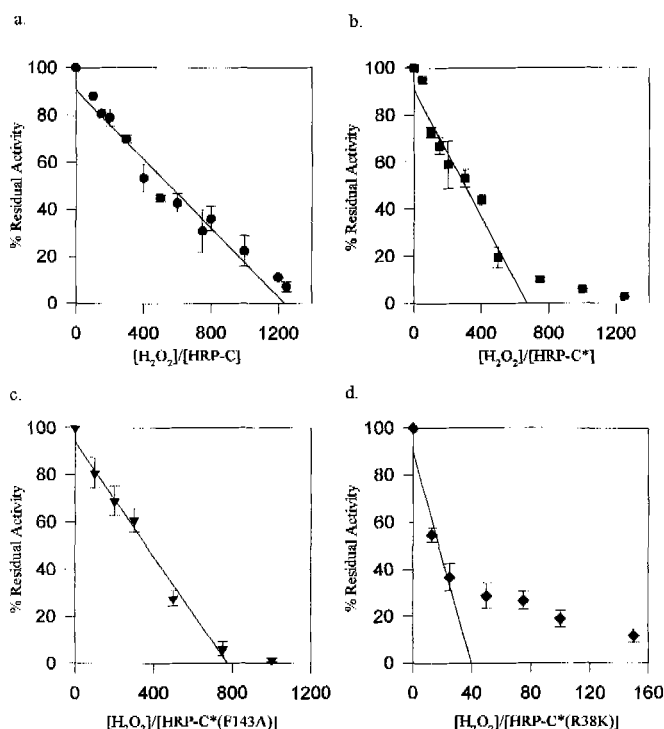


**Fig. 1.** Sensitivity to inactivation of each enzyme at different molar ratios of  $\text{H}_2\text{O}_2$ . (a) HRP-C; (b) HRP-C\*; (c) [F143A]HRP-C\*; (d) [R38K]HRP-C\*. Reactions were incubated in 0.2 ml 50 mM sodium phosphate pH 6.5 containing 0.25  $\mu\text{M}$  enzyme over a range of substrate concentrations. Residual activity was measured using ABTS after 4 h. Lines were fitted using Sigmaplot for Windows. The values of the intercepts at the x-axis were used to calculate the values of  $r$  (see text and Table 1).

**Table 1.** Values of  $r$  (turnover) for inactivation with  $\text{H}_2\text{O}_2$  and  $m\text{ClO}_2\text{BzOH}$ . Values were calculated from plots as shown in Fig. 1 using the procedure described in the results and discussion.

| Enzyme        | $r$ for                |                            |
|---------------|------------------------|----------------------------|
|               | $\text{H}_2\text{O}_2$ | $m\text{ClO}_2\text{BzOH}$ |
| HRP-C         | 625                    | 2.6                        |
| HRP-C*        | 335                    | 2.5                        |
| [F143A]HRP-C* | 385                    | 3.0                        |
| [R38K]HRP-C*  | 20                     | 3.1                        |

**Isoelectric focussing.** Isoelectric focussing was performed on a PhastSystem (Pharmacia-LKB) using PhastGel IEF 3-9 prepared gels and the broad-pI calibration kit (3.50–9.30) (Pharmacia-LKB). This kit contained the following proteins with the corresponding pI values: lentil lectin, basic (8.65); lentil lectin, middle (8.45); lentil lectin, acidic (8.15); horse myoglobin, basic (7.35); horse myoglobin, acidic (6.85); human carbonic anhydrase B (6.55); bovine carbonic anhydrase B (5.85);  $\beta$ -lactoglobulin A (5.20); soybean trypsin inhibitor (4.55); amyloglucosidase (3.50).

The proteins were stained using the silver stain kit (Pharmacia-LKB) following the method of development given. The band evaluations and the pI determinations were made using the PhastImage gel analyzer and PhastImage software v. 1.0 (Pharmacia-LKB).

For peroxidase activity staining, PhastGels were incubated in 25-ml solutions containing 1 mM 4-methoxy-1-naphthol,

0.4 mM H<sub>2</sub>O<sub>2</sub> in 50 mM Tris/HCl pH 7.5 at 4°C, for 15 min, in the dark [43]. The stained gels were analyzed using the Phast-Image gel analyzer.

## RESULTS AND DISCUSSION

The enzymic activity of recombinant unglycosylated horseradish peroxidase measured with ABTS is similar to that of the plant enzyme as noted previously [27]. Despite the presence of an additional amino-terminal Met in the former, both enzymes have been shown to be highly similar in activity [27], NMR [38] and resonance Raman [44] spectroscopic measurements. In contrast, [F143A]HRP-C\* exhibited increased activity whilst [R38K]HRP-C\* showed greatly reduced activity. Examination of these results revealed a number of interesting differences between the enzymes. The mutation of the bulky Phe residue into a much smaller Ala in [F143A]HRP-C\* increased the activity with ABTS. This may be due to improved access for the reductant to the active site or a change in the product dissociation rate, as has been observed with some other mutants [28, 45]. [R38K]HRP-C\* is known to have lowered affinity for H<sub>2</sub>O<sub>2</sub>, a slower (rate-limiting) and partially reversible reaction with the oxidant to produce compound I, and reduced stability of this intermediate [33]; the effect on activity with ABTS is a global reduction in rate mediated by the formation of compound I.

The differences observed in the presence of reductant substrate along with H<sub>2</sub>O<sub>2</sub> as the oxidant provided a justification for a detailed examination of the effect of using H<sub>2</sub>O<sub>2</sub> as both oxidant and reductant substrate which, when provided in excess, has been shown to inactivate HRP-C [25].

Incubation of the enzymes with different ratios of excess H<sub>2</sub>O<sub>2</sub> followed by the determination of the residual activity with ABTS at the end of the reactions permitted the susceptibility of the different variants to be measured in an easily comparable way. The values of the intercepts at the x-axis (molar ratio of H<sub>2</sub>O<sub>2</sub> required for 100% inactivation) are shown in Fig. 1. The values of *r* (number of turnovers of H<sub>2</sub>O<sub>2</sub> by the enzyme before inactivation) were calculated in accordance with [46] using the equation:

$$A_r = \frac{A_t}{A_0} = 1 - \frac{1}{1+r} \cdot \frac{[\text{ROOH}]}{[\text{HRP}]} \quad (1)$$

where *A<sub>r</sub>* is the residual activity; *A<sub>t</sub>* and *A<sub>0</sub>* are the activities at time *t* (end of reaction) and zero, respectively; *r* is the number of turnovers; [ROOH] and [HRP] are the initial concentrations of peroxide and enzyme. Taking into account the consumption of two moles H<sub>2</sub>O<sub>2</sub> in each catalytic cycle (one mole for compound I formation and another for inactivation or catalysis) [25], the values of *r* obtained and summarised in Table 1, were 625 (HRP-C), 335 (HRP-C\*), 385 ([F143A]HRP-C\*) and 20 ([R38K]HRP-C\*). Thus HRP-C was most resistant to inactivation, HRP-C\* and [F143A]HRP-C\* mutant were about twice as sensitive and [R38K]HRP-C\* was very much more sensitive. It is not clear why HRP-C\* was more sensitive to H<sub>2</sub>O<sub>2</sub> than plant HRP-C. However a similar increase in sensitivity has previously been seen in partially deglycosylated avocado peroxidase [47], and a protective role for the sugar residues of the wild-type plant proteins has been suggested [48]. Deviation from linearity was observed for [R38K]HRP-C\* for molar ratios of H<sub>2</sub>O<sub>2</sub> of approximately 30 and above where the level of residual activity was around 30% or less. This was either due to a mechanistic change in the process of inactivation for this mutant or to the presence of an inactive low-spin form of [R38K]HRP-C\* (termed the I<sup>-</sup> form in [32]) in slow equilibrium with the active form. Sanders et al. [32] have suggested that approximately 40% of [R38K]HRP-C\* may be in the I<sup>-</sup> form at pH 6.5. It is appar-

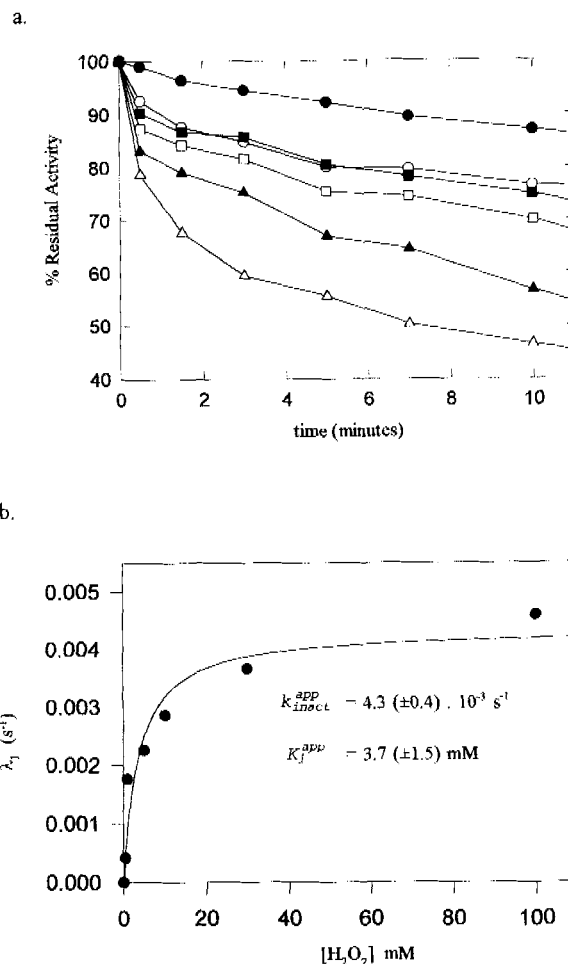


**Fig. 2. Time dependence and kinetics of the inactivation of HRP-C\* by H<sub>2</sub>O<sub>2</sub>.** (a) Inactivation of HRP-C\* followed against time. Enzyme (0.5 μM) was incubated with H<sub>2</sub>O<sub>2</sub> at mM concentrations of (●) 0.5, (○) 1.0, (■) 5.0, (□) 10.0, (▲) 30.0 and (△) 100; 10-μl aliquots were assayed for residual activity using ABTS at the specified times. (b) Plot of  $\lambda_1$  against H<sub>2</sub>O<sub>2</sub> concentration. The apparent rate constant of inactivation,  $k_{inact}^{app}$ , and the dissociation constant,  $K_i^{app}$ , were obtained using Sigmaplot for Windows.

ent that the R38K mutation renders this variant considerably more sensitive to H<sub>2</sub>O<sub>2</sub> than is the case for [F143A]HRP-C\* which behaved in essentially the same way as the recombinant enzyme under the same conditions.

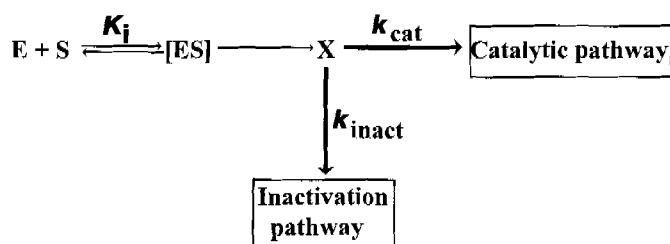
The xenobiotic peroxidase substrate *m*ClO<sub>2</sub>BzOH has been previously identified as a potent inactivator of wild-type HRP-C [26]. Incubation of the recombinant and mutants with a range of ratios of *m*ClO<sub>2</sub>BzOH/enzyme yielded very similar values of *r* (Table 1) to the plant enzyme, ranging from 2.5 to 3.1 for 100% inactivation of the sample, indicating that this inactivator was equally potent with all the enzymes. This suggests that its mode of action is in some way distinct from that of H<sub>2</sub>O<sub>2</sub> and must relate to the ability of H<sub>2</sub>O<sub>2</sub> to function both as an oxidant and a reductant. Because of the similarity of all these results, the effects of *m*ClO<sub>2</sub>BzOH were not investigated further in the present work, although a careful kinetic study has been developed for the inactivation of wild-type HRP-C by *m*ClO<sub>2</sub>BzOH [49].

The inactivation of each of the enzymes by H<sub>2</sub>O<sub>2</sub> at a range of concentrations was also followed with time (the results for HRP-C\* which were representative of those obtained for each enzyme are shown in Fig. 2a). Double-exponential decays in the

**Table 2. Apparent kinetic constants calculated for the inactivation of peroxidase variants by  $\text{H}_2\text{O}_2$ .**  $k_{\text{inact}}^{\text{app}}$ , rate constant of inactivation;  $K_1^{\text{app}}$ , dissociation constant of the enzyme substrate complex, ES;  $1/K_1^{\text{app}}$ , affinity constant of enzyme and  $\text{H}_2\text{O}_2$ ;  $k_{\text{cat}}^{\text{app}}$ , rate constant of catalysis;  $k_{\text{cat}}^{\text{app}}/K_1^{\text{app}}$ , efficiency of catalysis;  $k_{\text{inact}}^{\text{app}}/K_1^{\text{app}}$ , efficiency of inactivation;  $\lambda_2$ , inactivation constant for the slowest phase (Eqn. 2). Values of  $k_{\text{inact}}^{\text{app}}$ ,  $K_1^{\text{app}}$  and  $\lambda_2$  are means  $\pm$  error.

| Enzyme        | $10^3 \times k_{\text{inact}}^{\text{app}}$<br>s <sup>-1</sup> | $K_1^{\text{app}}$<br>mM | $1/K_1^{\text{app}}$<br>M <sup>-1</sup> | $k_{\text{cat}}^{\text{app}}$<br>s <sup>-1</sup> | $k_{\text{cat}}^{\text{app}}/K_1^{\text{app}}$<br>s <sup>-1</sup> M <sup>-1</sup> | $k_{\text{inact}}^{\text{app}}/K_1^{\text{app}}$ | $10^3 \times \lambda_2$<br>s <sup>-1</sup> |
|---------------|----------------------------------------------------------------|--------------------------|-----------------------------------------|--------------------------------------------------|-----------------------------------------------------------------------------------|--------------------------------------------------|--------------------------------------------|
| HRP-C         | 4.6 $\pm$ 0.2                                                  | 1.3 $\pm$ 0.2            | 775                                     | 2.9                                              | 2248                                                                              | 3.5                                              | 0.3 $\pm$ 0.02                             |
| HRP-C*        | 4.3 $\pm$ 0.4                                                  | 3.7 $\pm$ 1.4            | 238                                     | 1.5                                              | 397                                                                               | 1.2                                              | 0.3 $\pm$ 0.07                             |
| [F143A]HRP-C* | 3.5 $\pm$ 0.2                                                  | 5.2 $\pm$ 1.0            | 193                                     | 1.4                                              | 260                                                                               | 0.7                                              | 0.3 $\pm$ 0.06                             |
| [R38K]HRP-C*  | 8.9 $\pm$ 1.0                                                  | 6.3 $\pm$ 2.5            | 160                                     | 0.2                                              | 29                                                                                | 1.4                                              | 0.4 $\pm$ 0.1                              |

**Scheme 1. A minimal scheme describing the action of a mechanism-based inactivator (suicide substrate).** For the reaction under study, the species represented are: E, compound I; S, peroxide; ES, compound I–peroxide complex; X, compound I–peroxide modified complex (transition state).  $K_1$  is the dissociation constant of ES;  $k_{\text{inact}}$ , the apparent rate constant of inactivation;  $k_{\text{cat}}$ , the apparent rate constant of catalysis.



enzymic activity were seen. Such behaviour has been previously described using HRP-C from various sources [25, 50]. The observed pseudo-first-order rate constants of inactivation are described by the equation:

$$\frac{A_t}{A_0} = \gamma_1 e^{-\lambda_1 t} + \gamma_2 e^{-\lambda_2 t} \quad (2)$$

where  $A_t$  and  $A_0$  are the measures of peroxidase activity at incubation times of  $t$  and zero;  $\lambda_1$  and  $\lambda_2$  are the apparent inactivation constants of the system; and  $\gamma_1$  and  $\gamma_2$  are the amplitudes of the exponential terms. An alternative simplified form of Eqn (2) if one considers that  $\gamma_1 + \gamma_2 = 1$ :

$$\frac{A_t}{A_0} = (1 - \gamma_2) e^{-\lambda_1 t} + \gamma_2 e^{-\lambda_2 t} \quad (3)$$

In this case, only three parameters ( $\gamma_2$ ,  $\lambda_1$  and  $\lambda_2$ ) appear.

Double-exponential fits were made to the data to obtain the apparent inactivation constants  $\lambda_1$  and  $\lambda_2$ . Plotting the values of  $\lambda_1$  against the  $\text{H}_2\text{O}_2$  concentration produced a hyperbolic curve from which it was clear that the inactivation process exhibited saturation kinetics (Fig. 2b). However, the values for  $\lambda_2$  were independent of the  $\text{H}_2\text{O}_2$  concentration, one order of magnitude lower than those for  $\lambda_1$  and very similar for all the samples tested (Table 2). Therefore, we did not consider further the contribution of  $\lambda_2$  in the comparative study.

The minimal model capable of describing the observed reaction is given in Scheme 1, a model describing the action of a suicide substrate (mechanism-based inhibitor) [51–53]. The general scheme was applied to the present reaction in the following way. The reaction of ferric HRP with peroxide to form compound I was not taken into account in the model because this reaction occurs at a very much faster rate than the overall processes of inactivation or catalytic turnover; E in Scheme 1 is therefore compound I, and ES the complex between compound

I and a second molecule of peroxide, [compound I ·  $\text{H}_2\text{O}_2$ ]. X represents a modified complex or transition state, [compound I ·  $\text{H}_2\text{O}_2$ ]\*, required by the kinetic model from which the enzyme can follow either an inactivatory pathway, or the protective catalytic turnover of substrate to generate products and active enzyme. The reaction proceeds in the transition phase until all the enzyme is inactivated. The model is in fact a simplification of the full kinetic approach developed previously by us [25], but it offers a reasonable approximation suitable for comparative purposes as required here. Double-reciprocal plots of the rate of inhibition against inhibitor concentration have often been used to examine the formation of complexes between enzymes and inhibitors [50, 54, 55]. We used non-linear least-squares fits to the data (Fig. 2b) to determine the relationship between the different enzymes and  $\text{H}_2\text{O}_2$ . We have obtained for each variant the apparent values of the rate constant of inactivation ( $k_{\text{inact}}^{\text{app}}$ ) and the dissociation constant ( $K_1^{\text{app}}$ ) of the enzyme/inhibitor complex, and in conjunction with the turnover numbers ( $r$ ) above, the values of the catalytic constant ( $k_{\text{cat}}^{\text{app}}$ ) using the relationship:

$$r = \frac{k_{\text{cat}}^{\text{app}}}{k_{\text{inact}}^{\text{app}}} \quad (4)$$

The results obtained are given in Table 2 and summarised below.

The value of  $k_{\text{inact}}^{\text{app}}$  with  $\text{H}_2\text{O}_2$  was very similar for HRP-C, HRP-C\* and [F143A]HRP-C\* but the compound I of HRP-C had the highest affinity for  $\text{H}_2\text{O}_2$  ( $1/K_1^{\text{app}}$ ), rate constant for catalysis ( $k_{\text{cat}}^{\text{app}}$ ), efficiency of catalysis ( $k_{\text{cat}}^{\text{app}}/K_1^{\text{app}}$ ) and also efficiency of inactivation ( $k_{\text{inact}}^{\text{app}}/K_1^{\text{app}}$ ). HRP-C\* was inactivated at about the same rate as HRP-C and all the other parameters described above were lower by a factor of 2–3 except for  $k_{\text{cat}}^{\text{app}}/K_1^{\text{app}}$  which was reduced about sixfold. [F143A]HRP-C\* had a lower  $k_{\text{inact}}^{\text{app}}/K_1^{\text{app}}$  in conjunction with a lower affinity of its compound I for peroxide, but a  $k_{\text{cat}}^{\text{app}}$  comparable to that of HRP-C\*. The  $k_{\text{inact}}^{\text{app}}$  and  $K_1^{\text{app}}$  of [R38K]HRP-C\* were 2-fold and 5-fold higher, respectively, than those of HRP-C, the  $k_{\text{cat}}^{\text{app}}$  and  $k_{\text{cat}}^{\text{app}}/K_1^{\text{app}}$  15-fold and 75-fold lower, respectively, the efficiency of inactivation however was half that of HRP-C.

Comparing the two mutants, [F143A]HRP-C\* and [R38K]HRP-C\*, which have comparable values of affinity for  $\text{H}_2\text{O}_2$ , it is seen that the coupling of a high  $k_{\text{inact}}^{\text{app}}$  with low  $k_{\text{cat}}^{\text{app}}$  has exposed [R38K]HRP-C\* to inactivation, whereas [F143A]HRP-C\* is protected from inactivation by virtue of its higher catalytic constant ( $k_{\text{cat}}^{\text{app}}$ ) which allows the enzyme to turnover  $\text{H}_2\text{O}_2$  rather than progress down the inactivatory pathway. In the case of HRP-C the high affinity of compound I for peroxide with similarly high values of  $k_{\text{inact}}^{\text{app}}$  and  $k_{\text{cat}}^{\text{app}}/K_1^{\text{app}}$  have given it the greatest efficiency of inactivation. The rate of inactivation is comparable to that obtained for HRP-C\* or [F143A]HRP-C\*, but the greatest number of turnovers (highest  $r$ ) of substrate are made by the enzyme before it is inactivated.

It is concluded that structural and mechanistic perturbations induced by the mutation in [R38K]HRP-C\* have a dramatic effect on the partitioning of the [compound I · H<sub>2</sub>O<sub>2</sub>]\* complex (Scheme 1) between inactivation and catalysis. Catalytic activity was reduced and the enzyme made more sensitive to inactivation by H<sub>2</sub>O<sub>2</sub>. However, residue changes which improve these parameters, i.e. increase activity or reduce sensitivity, could become evident with further work.

The accessibility to substrate of the reductant binding site in the haem pocket or its affinity for the product is important for enzyme activity as was observed with [F143A]HRP-C\*. Substrate specificity can be altered by changes affecting reductant binding [28] and it may be possible to adjust further the specificity and level of activity of HRP by judicious changes in this region.

The glycosylation of wild-type HRP-C appears to be significant in protecting the enzyme from inactivation but does not affect the activity of HRP-C\*. The physiological significance to the susceptibility of HRP-C to inactivation by H<sub>2</sub>O<sub>2</sub> will remain unclear until more is known about the physical changes which are induced by the process. However, the native enzyme is the most active in the normal peroxidase cycle, suggesting that the active site of the enzyme had evolved not only for improving catalytic efficiency but also for impeding inactivation by the highly reactive H<sub>2</sub>O<sub>2</sub> substrate.

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## REFERENCES

1. Welinder, K. G. (1992) Superfamily of plant, fungal and bacterial peroxidases, *Curr. Opin. Struct. Biol.* **2**, 388–393.
2. Welinder, K. G. (1985) Plant peroxidases. Their primary, secondary and tertiary structures, and relation to cytochrome *c* peroxidase, *Eur. J. Biochem.* **151**, 497–504.
3. Shannon, L. M., Kay, E. & Lew, J. Y. (1966) Peroxidase isoenzymes from horseradish roots. I. Isolation and physical properties, *J. Biol. Chem.* **241**, 2166–2172.
4. Dunford, H. B. & Stillman, J. S. (1976) On the function and mechanism of action of peroxidases, *Coord. Chem. Rev.* **19**, 187–251.
5. Haschke, R. H. & Friedhoff, J. M. (1978) Calcium-related properties of horseradish peroxidase, *Biochem. Biophys. Res. Commun.* **80**, 1039–1042.
6. Shiro, Y., Kurono, M. & Morishima, I. (1986) Presence of endogenous calcium ion and its functional and structural regulation in horseradish peroxidase, *J. Biol. Chem.* **261**, 9382–9390.
7. Ogawa, S., Shiro, Y. & Morishima, I. (1979) Calcium binding by horseradish peroxidase C and the heme environmental structure, *Biochem. Biophys. Res. Commun.* **90**, 674–678.
8. Morishima, I., Kurono, M. & Shiro, Y. (1986) Presence of endogenous calcium ion in horseradish peroxidase, *J. Biol. Chem.* **261**, 9391–9399.
9. Morita, Y., Mikami, B., Yamashita, H., Lee, J. Y., Aibara, S., Sato, M., Katsube, Y. & Tanaka, N. (1991) Primary and crystal structure of horseradish peroxidase isozyme E5, in *Biochemical, molecular and physiological aspects of plant peroxidases* (Lobazewski, J., Greppin, H., Penel, C. & Gaspar, T., eds) pp. 81–88, University of Geneva, Geneva.
10. Finzel, B. C., Poulos, T. L. & Kraut, J. (1984) Crystal structure of yeast cytochrome *c* peroxidase refined at 1.7 Å resolution, *J. Biol. Chem.* **259**, 13027–13036.
11. Welinder, K. G. & Nørskov-Lauritsen, L. (1986) Structure of plant peroxidases – Preliminary fitting into the molecular model of yeast cytochrome *c* peroxidase, in *Molecular and physiological aspects of plant peroxidases* (Greppin, H., Penel, C. & Gaspar, T., eds) pp. 61–70, University of Geneva, Geneva.
12. Ator, M. A. & Ortiz de Montellano, P. R. (1987) Protein control of prosthetic group reactivity. Reaction of substrates with the heme edge of horseradish peroxidase, *J. Biol. Chem.* **262**, 1542–1551.
13. Penner-Hahn, J. E., Elde, K. S., McMurry, T. J., Rinner, M., Balch, A. L., Groves, J. J., Dawson, J. H. & Hodgson, K. O. (1986) Structural characteristics of horseradish peroxidase using EXAFS spectroscopy. Evidence for Fe=O ligation in compounds I and II, *J. Am. Chem. Soc.* **108**, 7819–7825.
14. Sivaraja, M., Goodin, D. B., Smith, M. & Hoffman, B. M. (1990) Identification by ENDOR of Trp191 as the free-radical site in cytochrome *c* peroxidase compound ES, *Science* **245**, 738–740.
15. Hassinoff, B. B. & Dunford, H. B. (1970) Kinetics of the oxidation of ferrocyanide by horseradish peroxidase compounds I and II, *Biochemistry* **9**, 4930–4939.
16. Job, D. & Dunford, H. B. (1976) Substituent effect on the oxidation of phenols and aromatic amines by horseradish peroxidase compound I, *Eur. J. Biochem.* **66**, 607–614.
17. Kobayashi, S., Nakano, M., Goto, T., Kimura, T. & Schaap, A. P. (1986) An evidence of the peroxidase-dependent oxygen transfer from hydrogen peroxide to sulfides, *Biochem. Biophys. Res. Commun.* **135**, 166–171.
18. Kobayashi, S., Nakano, M., Kimura, T. & Schaap, A. P. (1987) On the mechanism of the peroxidase-catalyzed oxygen-transfer reaction, *Biochemistry* **26**, 5019–5022.
19. Doerge, D. R. (1986) Oxygenation of organosulfur compounds by peroxidases: evidence of an electron transfer mechanism for lactoperoxidase, *Arch. Biochem. Biophys.* **244**, 678–685.
20. Doerge, D. R., Cooray, N. M. & Brewster, M. E. (1991) Peroxidase-catalyzed S-oxidation: mechanism of oxygen transfer for lactoperoxidase, *Biochemistry* **30**, 8960–8964.
21. Keilin, D. & Hartree, E. F. (1951) Purification of horseradish peroxidase and comparison of its properties with those of catalase and methaemoglobin, *Biochem. J.* **49**, 88–104.
22. Nicholls, P. & Schonbaum, G. R. (1963) Catalases, in *The enzymes*, vol. 8 (Boyer, P. D., Lardy, H. & Myrback, K., eds) 1st edn, pp. 147–225, Academic Press, New York.
23. Nakajima, R. & Yamazaki, I. (1987) The mechanism of oxyperoxidase formation from ferryl peroxidase and hydrogen peroxide, *J. Biol. Chem.* **262**, 2576–2581.
24. Nakajima, R. & Yamazaki, I. (1980) The conversion of horseradish peroxidase C to a verdohemoprotein by a hydroperoxide derived enzymatically from indole-3-acetic acid and by *m*-nitroperoxybenzoic acid, *J. Biol. Chem.* **255**, 2067–2072.
25. Arnao, M. B., Acosta, M., Del Río, J. A., Varón, R. & García-Cánovas, F. (1990) A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide, *Biochim. Biophys. Acta* **1041**, 43–47.
26. Acosta, M., Arnao, M. B., Del Río, J. A. & García-Cánovas, F. (1991) Inactivation of peroxidases, in *Biochemical, molecular and physiological aspects of plant peroxidases* (Lobazewski, J., Greppin, H., Penel, C. & Gaspar, T., eds) pp. 175–184, University of Geneva, Geneva.
27. Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C., Thorneley, R. N. F. & Burke, J. F. (1990) Expression of a synthetic gene for horseradish peroxidase C in *Escherichia coli* and folding and activation of the recombinant enzyme with Ca<sup>2+</sup> and heme, *J. Biol. Chem.* **265**, 13335–13343.
28. Smith, A. T., Sanders, S. A., Thorneley, R. N. F., Burke, J. F. & Bray, R. R. C. (1992) Characterisation of a haem active-site mutant of horseradish peroxidase, Phe41→Val, with altered reactivity towards hydrogen peroxide and reducing substrates, *Eur. J. Biochem.* **207**, 507–519.
29. Poulos, T. L. & Kraut, J. (1980) The stereochemistry of peroxidase catalysis, *J. Biol. Chem.* **255**, 8199–8205.
30. Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J. & Kraut, J. (1993) Effect of arginine-48 replacement on the reaction between cytochrome *c* peroxidase and hydrogen peroxide, *Biochemistry* **32**, 9807–9818.
31. Erman, J. E. & Vitello, L. B. (1993) The effect of active-site mutations in cytochrome *c* peroxidase on the rate of compound I formation, in *Plant peroxidases: Biochemistry and physiology*

- (Welinder, K. G., Rasmussen, S. K., Penel, C. & Greppin, H., eds) pp. 149–154, University of Geneva, Geneva.
32. Sanders, S. A., Bray, R. C. & Smith, A. T. (1994) pH-dependent properties of a mutant horseradish peroxidase isoenzyme C in which Arg38 has been replaced with lysine, *Eur. J. Biochem.* **224**, 1029–1037.
33. Smith, A. T., Sanders, S. A., Sampson, C., Bray, R. C., Burke, J. F. & Thorneley, R. N. F. (1993) Folding and activation of recombinant horseradish peroxidase from *E. coli* and analysis of protein variants produced by site-directed mutagenesis, in *Plant peroxidases: Biochemistry and physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C. & Greppin, H., eds) pp. 159–168, University of Geneva, Geneva.
34. Veitch, N. C. & Williams, R. J. P. (1990) Two-dimensional <sup>1</sup>H-NMR studies of horseradish peroxidase C and its interaction with indole-3-propionic acid, *Eur. J. Biochem.* **189**, 351–362.
35. Veitch, N. C. (1993) NMR studies of the substrate binding site in plant and fungal peroxidases, in *Plant peroxidases: Biochemistry and physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C. & Greppin, H., eds) pp. 57–64, University of Geneva, Geneva.
36. Veitch, N. C., Williams, R. J. B., Bone, N. M., Burke, J. F. & Smith, A. T. (1995) Solution characterisation by NMR spectroscopy of two horseradish peroxidase isoenzyme C mutants with alanine replacing Phe142 or Phe143, *Eur. J. Biochem.* **233**, 650–658.
37. Nakajima, R. & Yamazaki, I. (1979) The mechanism of indole-3-acetic acid oxidation by horseradish peroxidases, *J. Biol. Chem.* **254**, 872–878.
38. Veitch, N. C., Williams, R. J. P., Bray, R. C., Burke, J. F., Sanders, S. A., Thorneley, R. N. F. & Smith, A. T. (1992) Structural studies by proton-NMR spectroscopy of plant horseradish peroxidase C, the wild-type recombinant protein from *Escherichia coli* and two protein variants, Phe41→Val and Arg38→Lys, *Eur. J. Biochem.* **207**, 521–531.
39. Pourchert, C. J. & Campbell, J. R. (1974) in *The Aldrich library of NMR spectra*, vol. 6, p. 146, Aldrich Chemical Company, Milwaukee WI.
40. Davies, D. M., Jones, P. & Mantle, D. (1976) The kinetics of formation of horseradish peroxidase compound I by reaction with peroxobenzoic acids. pH and peroxo acid substituent effects, *Biochem. J.* **157**, 247–253.
41. Childs, R. E. & Bardsley, W. G. (1975) The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) as chromogen, *Biochem. J.* **145**, 93–103.
42. Bielski, B. H. J. & Allen, A. O. (1977) Mechanism of the disproportion of superoxide radicals, *J. Phys. Chem.* **81**, 1048–1050.
43. Guilbault, G. G. & Kramer, D. N. (1964) 4-Methoxy-*α*-naphthol as a spectrophotometric reagent substrate for measuring peroxidatic activity, *Anal. Chem.* **36**, 2494–2496.
44. Smulevich, G., Burke, J. F., Padi, M., Sanders, S., Thorneley, R. N. F. & Smith, A. T. (1994) Characterisation of recombinant horseradish peroxidase (HRP-C\*) and three site-directed mutants F41V, F41W, and R38K by resonance raman spectroscopy, *Biochemistry* **33**, 7398–7406.
45. Gazaryan, I. G., Doseeva, V. V., Galkin, A. G. & Tishkov, V. I. (1994) Effect of single-point mutations Phe41→His and Phe143→Glu on folding and catalytic properties of recombinant horseradish peroxidase expressed in *E. coli*, *FEBS Lett.* **354**, 248–250.
46. García-Cánovas, F., Tudela, J., Varón, R. & Vazquez, A. M. (1989) Experimental methods for kinetic study of suicide substrates, *J. Enz. Inhib.* **3**, 81–90.
47. Sánchez-Romero, C., García-Gómez, M. L., Pliego-Alfaro, F. & Heredia, A. (1994) Effect of partial deglycosylation on catalytic characteristics and stability of an avocado peroxidase, *Physiol. Plant.* **92**, 97–101.
48. Lambeir, A., Dunford, H. B., Van Huystee, R. B. & Lobarzewski, J. (1985) Spectral and kinetic properties of a cationic peroxidase secreted by cultured peanut cells, *Can. J. Biochem. Cell Biol.* **63**, 1086–1092.
49. Arnao, M. B., Hernández-Ruiz, J., Varón, R., García-Cánovas, F. & Acosta, M. (1995) The inactivation of horseradish peroxidase by *m*-chloroperoxybenzoic acid, a xenobiotic hydroperoxide, *J. Mol. Catal.*, in the press.
50. Baynton, K. J., Bewtra, J. K., Biswas, N. & Taylor, K. E. (1994) Inactivation of horseradish peroxidase by phenol and hydrogen peroxide: a kinetic investigation, *Biochim. Biophys. Acta* **1206**, 272–278.
51. Walsh, C. T. (1984) Suicide substrates, mechanism-based enzyme inactivators: recent developments, *Annu. Rev. Biochem.* **53**, 493–535.
52. Waley, S. G. (1980) Kinetics of suicide substrates, *Biochem. J.* **185**, 771–773.
53. Waley, S. G. (1985) Kinetics of suicide substrates, *Biochem. J.* **227**, 843–849.
54. Walsh, C. T. (1979) *Enzymatic reaction mechanisms*, W. H. Freeman, San Francisco.
55. Ator, M. A., David, S. K. & Ortiz de Montellano, P. R. (1987) Structure and catalytic mechanism of horseradish peroxidase. Regio-specific *meso* alkylation of the prosthetic group by alkylhydrazines, *J. Biol. Chem.* **262**, 14954–14960.