Kinetic study of the inactivation of ascorbate peroxidase by hydrogen peroxide

Alexander N. P. HINER*, José Neptuno RODRÍGUEZ-LÓPEZ†, Marino B. ARNAO*, Emma LLOYD RAVEN‡, Francisco GARCÍA-CÁNOVAS† and Manuel ACOSTA*¹

*Departamento de Biología Vegetal (Fisiología Vegetal), Universidad de Murcia, E-30100 Espinardo, Murcia, Spain,

†Departamento de Bioquímica y Biología Molecular-A, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain, and

‡Department of Chemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K.

The activity of ascorbate peroxidase (APX) has been studied with H_2O_2 and various reducing substrates. The activity decreased in the order pyrogallol > ascorbate > guaiacol > 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). The inactivation of APX with H_2O_2 as the sole substrate was studied. The number of H_2O_2 molecules required for maximal inactivation of the enzyme was determined as approx. 2.5. Enzymic activity of approx. 20 % of the original remained at the end of the inactivation process (i.e. approx. 20 % resistance) when ascorbate or ABTS was used as the substrate in activity assays. With pyrogallol or guaiacol no resistance was seen. Inactivation by H_2O_2 followed over time with ascorbate or pyrogallol assays exhibited single-exponential decreases in enzymic activity. Hyperbolic saturation kinetics were observed in both assay systems; a similar dissociation constant ($0.8 \mu\text{M}$) for H_2O_2 was obtained in each case. However, the maximum rate constant (λ_{max}) obtained from the plots differed depending on the assay substrate.

The presence of reducing substrate in addition to H_2O_2 partly or completely protected the enzyme from inactivation, depending on how many molar equivalents of reducing substrate were added. An oxygen electrode system has been used to confirm that APX does not exhibit a catalase-like oxygen-releasing reaction. A kinetic model was developed to interpret the experimental results; both the results and the model are compared and contrasted with previously obtained results for horseradish peroxidase C. The kinetic model has led us to the conclusion that the inactivation of APX by H_2O_2 represents an unusual situation in which no enzyme turnover occurs but there is a partition of the enzyme between two forms, one inactive and the other with activity towards reducing substrates such as ascorbate and ABTS only. The partition ratio is less than 1.

Key words: catalase, mechanism-based inactivation, protection, reductants, suicide inactivation.

INTRODUCTION

The superfamily of haem peroxidases from plants, fungi and bacteria are a group of enzymes that utilize H_2O_2 to oxidize a second (reducing) substrate. These enzymes share a similar catalytic cycle in which H_2O_2 reacts with the resting ferric enzyme to form the intermediate compound I (known as compound ES in cytochrome *c* peroxidase) which carries two oxidizing equivalents. Compound I is subsequently reduced by reactions with two reducing substrate molecules. The first of these reduction steps generates the intermediate, compound II, which is then further reduced back to the ferric enzyme.

Haem peroxidases have been classified into three classes [1]: class I contains intracellular enzymes [e.g. yeast cytochrome *c* peroxidase (CcP), ascorbate peroxidase (APX) and bacterial gene-duplicated catalase-peroxidases] [2]; class II consists of secretory fungal enzymes (e.g. manganese peroxidase and lignin peroxidase); class III contains the secretory plant peroxidases [e.g. horseradish peroxidase (HRP)]. The classification is based on sequence comparisons and enzyme localization rather than on function; thus APX, which preferentially reacts with a small substrate molecule, ascorbate, is in the same class as CcP, which reacts with another protein, cytochrome *c*.

APX (EC 1.11.1.11) is located in the chloroplasts and cytosol of plants and eukaryotic algae, and has also been identified in certain cyanobacteria [3]. In the chloroplasts, thylakoid-bound ('tAPX') [4,5] and soluble stromal ('sAPX') [6,7] forms have been

described. In the cytosol a single isoform was previously believed to be present [7] that exhibited slightly different characteristics from the chloroplastic enzymes [4–6]. However, by using the database of expressed sequence tags ('dbEST') from *Arabidopsis thaliana* (thale cress), seven APXs have been identified, including various soluble and membrane-bound cytosolic isoenzymes [8].

The function of all the forms of APX is thought to be the scavenging of the H_2O_2 that is continuously generated in cells [3,9]. For instance, in the chloroplasts of photosynthetic organisms superoxide ($\text{O}_2^{\cdot-}$) is formed when insufficient CO_2 is available to balance electrons being generated by the photosystems; these excess electrons then reduce O_2 to $\text{O}_2^{\cdot-}$. Additionally, in the mitochondria the electron transport chains can also produce $\text{O}_2^{\cdot-}$. In both cases superoxide dismutase converts $\text{O}_2^{\cdot-}$ into H_2O_2 , which APX or catalase can then remove.

We have previously studied the mechanism-based inactivation, by H_2O_2 and *m*-chloroperoxybenzoic acid (*m*-CPBA), of various isoenzymes and mutants of HRP [10–16]. We now present a similar examination of the inactivation of APX.

For this study we used the recombinant cytosolic form of pea APX [17]. The enzyme is a homodimer with a molecular mass of 57.5 kDa [17,18]. The structure has been determined by X-ray crystallography [18] and the cDNA encoding the enzyme has been sequenced [17]. APX shares 33 % sequence identity with CcP [17]. It has been observed that Trp-191 in CcP and the equivalent residue in APX, Trp-179, are conserved in identical conformations [18]. Trp-191 forms the site of the protein cation

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid); APX, ascorbate peroxidase; CcP, yeast cytochrome *c* peroxidase; [E], concentration of APX active sites; HR, hypersensitive response; HRP, horseradish peroxidase; *m*-CPBA, *m*-chloroperoxybenzoic acid; tAPX, thylakoid-bound APX.

¹ To whom correspondence should be addressed (e-mail macosta@fcu.um.es).

radical in CcP compound ES [19–21]. Most peroxidases, with the exception of CcP, are believed to form compound I species that possess a π -cation radical delocalized over the haem ring in addition to the oxyferryl iron centre common to all haem peroxidases [22]. However, recently evidence has emerged for the existence of a protein radical in wheat-germ peroxidase compound I [23]. In compound II the radical (whether π or protein) has been discharged by reaction with a molecule of reducing substrate to leave only the oxyferryl iron [24]. APX is generally thought to form a typical, but unstable, compound I containing a π -cation radical; this undergoes rapid conversion into another species, which exhibits a compound II-type absorption spectrum [25,26]. In our previous studies of the inactivation of HRP-C we have established that compound I has a central role in the reaction mechanism of inactivation, whereas compound II is much less important [16].

Because APX forms an important part of the defences of the cell against oxidative stress, the study of the inactivation of the enzyme by one of the major products of such stress, namely H_2O_2 , provides an indication of the limitations of resistance to attack by reactive oxygen species. Examination of the class I peroxidase, APX, also provides a useful comparison with the class III peroxidase, HRP, and extends our knowledge of peroxidase inactivation, providing an additional and distinct example of the process, within the established framework.

EXPERIMENTAL

APX

Recombinant pea cytosolic APX was expressed in and purified from *Escherichia coli* [17]. The enzyme used had an RZ value (A_{403}/A_{280}) of 1.93 and was homogeneous by gel electrophoresis. No peroxidase activity with 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ascorbate or guaiacol was detected in the sample after incubation with the specific APX inhibitor 4-chloromercuribenzoic acid [27], indicating that no guaiacol peroxidase was present. The concentration of enzyme was calculated from a molar absorption coefficient (ϵ) at 403 nm of $88 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ per haem. The individual active sites of APX are referred to as E throughout. Because APX is a homodimer, $[E] = 2[\text{APX}]$.

Chemicals

H_2O_2 (30%, v/v) was from Aldrich, its concentration being determined spectrophotometrically from $\epsilon_{240} = 43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$. H_2O_2 concentration was additionally checked by using the HRP-catalysed reaction with ABTS, 2 mol of ABTS radical ($\epsilon_{414} = 31\,100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) being formed from 1 mol of H_2O_2 . *m*-CPBA was purchased from Aldrich and was further purified by recrystallization from light petroleum (boiling range 40–60 °C)/diethyl ether (3:1, v/v) [28], purity was estimated by NMR spectroscopy as 99% [29]. Crystalline L-ascorbic acid was obtained from Scharlau. Pyrogallol (1,2,3-trihydroxybenzene) and guaiacol (2-methoxyphenol) were from Aldrich. ABTS as its ammonium salt was purchased from Sigma. Experiments were performed in sodium phosphate buffer (50 mM, pH 7.0). All solutions were prepared with water drawn from a Milli-Q system (Millipore).

UV/visible spectroscopy

UV/visible electronic absorption spectroscopy was performed in a Perkin-Elmer Lambda-2 spectrophotometer interfaced on-line to a compatible PC. The cuvette temperature was controlled at

25 ± 0.1 °C by a Haake D1G circulating bath with heater/cooler unit attached.

Oxygen measurements

Measurements of dissolved oxygen concentration were made with a Hansatech (Kings Lynn, Cambs., U.K.) oxygraph unit controlled from a PC. The oxygraph used a Clark-type silver/platinum electrode with a 0.0125 mm Teflon membrane. The sample was continuously stirred during experiments and its temperature was controlled at 25 °C, as above. The zero oxygen level for calibration and experiments was obtained by bubbling oxygen-free nitrogen through the sample for at least 10 min.

APX activity assays

APX activity was determined with assays containing H_2O_2 and one of the reducing substrates: ascorbate, pyrogallol, guaiacol or ABTS. For ascorbate the activity was followed as the decrease in A_{290} due to the consumption of ascorbate in an assay containing 10 mM H_2O_2 and 0.5 mM ascorbate ($\epsilon_{290} = 2800 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in buffer. The activity with pyrogallol (5 mM) was determined at 430 nm ($\epsilon_{430} = 2470 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and guaiacol (30 mM) at 470 nm ($\epsilon_{470} = 5570 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The increases in absorbance as a result of the formation of the oxidized products were measured at these wavelengths. The assays contained 10 mM H_2O_2 . The ABTS assay followed the formation of the ABTS radical at 414 nm ($\epsilon_{414} = 31\,100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in a solution containing 30 mM ABTS and 10 mM H_2O_2 . The total volume of each assay was 1 ml in a quartz cuvette with a 1 cm path length. All the assays were started by the addition of a sample of APX. The exact final concentration of E added varied for each individual experiment but a concentration between 10 and 50 nM generated a satisfactory linear absorbance change over the 2–5 min assay period (30 s for pyrogallol). Activity was calculated from the gradient of the change in absorbance with time (ΔA) divided by the molar absorption coefficient (ϵ).

Time dependence of inactivation

The inactivation of APX by H_2O_2 over time was followed by incubating APX ($[E] = 80 \text{ nM}$) with H_2O_2 (0, 1, 2, 5, 7.5 and 10 μM) in buffer (500 μl total volume). The reaction was initiated by addition of the enzyme. At the appropriate time points 50 μl aliquots were removed and their residual enzymic activities (A_R) were determined with the ascorbate or pyrogallol assay. The A_R was taken as the activity remaining (A_t) compared to the initial activity at the beginning of the experiment (A_0) expressed as a percentage. A plot of $\ln A_R$ against time permitted the calculation of the observed rate constants of inactivation (λ , s^{-1}). The maximum apparent rate constant of inactivation (λ_{max} , s^{-1}) and the dissociation constant of H_2O_2 from APX compound I [K_I (μM), the concentration of peroxide giving $\frac{1}{2}\lambda_{\text{max}}$] [30] were obtained by non-linear regression fitting of a hyperbolic curve to a plot of λ against $[\text{H}_2\text{O}_2]$. Curve and line fitting was done with the program SigmaPlot for Windows (Jandel Scientific Software, San Rafael, CA, U.S.A.).

Catalase activity of APX

Possible oxygen production as a result of a catalase-like action of APX on H_2O_2 was determined with the oxygraph on a total volume of 1.0 ml of deoxygenated buffer containing nominal H_2O_2 concentrations of 0, 0.1, 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 mM. The exact concentrations of H_2O_2 were determined spectroscopically just before use. The reaction was started by the addition of APX (0.25 μM final concentration of E). The initial

rate of oxygen production was calculated from the slope of the trace observed on the addition of APX after discounting the rapid rise in O_2 due to the introduction of sample containing dissolved air.

Determination of the number of equivalents of H_2O_2 required for APX inactivation

APX ($[E] = 0.30\text{--}0.50\ \mu\text{M}$, depending on each experiment) was incubated with molar ratios of H_2O_2 ($[H_2O_2]$ -to- $[E]$) ratios from 0 to 1000 were used, the actual concentrations of substrate added depending on the APX concentration) in buffer (100 μl total volume for each ratio). The inactivation process was left to continue to completion. After 16–18 h the residual APX activity (A_R) at each ratio was determined with the ascorbate, pyrogallol, guaiacol or ABTS assay. From plots of A_R against $[H_2O_2]/[E]$ a fitted line extrapolated from the linear part of the data to the x-axis gave the number of equivalents of H_2O_2 required for the inactivation of each active centre of the enzyme. For comparison a similar incubation experiment was performed in the presence of *m*-CPBA rather than H_2O_2 at $[m\text{-CPBA}]$ -to- $[E]$ ratios from 0 to 100, A_R was determined with the ascorbate assay (results not shown).

Protection of APX by reducing substrate against inactivation by H_2O_2

The same protocol as in the incubation experiments above was followed (0.25 μM APX) except that the incubation medium included ascorbate or guaiacol (1, 10 and 100 μM). Residual activities were determined with the ascorbate assay. Control incubations containing reducing substrate and H_2O_2 only showed that ascorbate was very slowly decomposed by H_2O_2 (followed at 290 nm). Guaiacol (observed at 470 nm) did not seem to be similarly sensitive.

RESULTS

Relative activities of APX with different reducing substrates

The activity of APX with a selection of substrates is shown in Table 1. The activity with ascorbate is defined as 1; thus pyrogallol was oxidized 3.2-fold more rapidly than ascorbate. Guaiacol and ABTS were poorer substrates of APX with 0.38-fold and 0.15-fold the activity of ascorbate respectively. The results given are values of $V_{\max}$; they are in good agreement with the relative activities of wild-type APX [31].

Time dependence and kinetics of APX inactivation by H_2O_2

When the decrease in residual activity due to inactivation by H_2O_2 was followed over time, with either the ascorbate or the

Figure 1 Time dependence and kinetics of the inactivation of APX by H_2O_2

(a) Inactivation of APX followed over time. APX ($[E] = 80\ \text{nM}$) was incubated with H_2O_2 at concentrations of 0 (●), 1.0 (■), 2.0 (▲), 5.0 (▼), 7.5 (◆) and 10.0 (●) μM in phosphate buffer (50 mM, pH 7.0, 25 °C). Aliquots of 50 μl were assayed at the specified times for residual activity with pyrogallol or ascorbate. (b) Plot with a natural logarithmic (ln) scale showing straight-line fits to the data in (a), from which the values of λ were determined. (c) Plot of λ against $[H_2O_2] - [E]$ with ascorbate (●) or pyrogallol (▲) assays. The apparent rate constants of inactivation ($\lambda_{\max}$) and the dissociation constants (K_i) were obtained by using SigmaPlot for Windows.

Table 1 Relative APX enzyme activities with different reducing substrates

Enzyme activities were determined spectroscopically with the assays described in the Experimental section; the relative activity with ascorbate was defined as 1. The concentrations of reagents used were: H_2O_2 , 10 mM; ascorbate, 0.5 mM; ABTS, 30 mM; pyrogallol, 5 mM; guaiacol, 30 mM in 50 mM sodium phosphate buffer, pH 7.0.

Reducing substrate in assay	$V_{\max}$ ($\text{M} \cdot \text{min}^{-1}$)	Relative activity
Ascorbate	5.3×10^{-5}	1.0
ABTS	8.2×10^{-6}	0.15
Pyrogallol	1.7×10^{-4}	3.2
Guaiacol	2.0×10^{-5}	0.38

pyrogallol assay, single-exponential curves were obtained for several hours (results for ascorbate are shown in Figure 1a). When the enzyme activity remaining was determined with the

Scheme 1 Proposed mechanism of the inactivation of APX by H_2O_2

E, APX active site; S, H_2O_2 ; E', enzyme compound formed by reaction of E and S; E'S, complex between E' and S; E_i, inactive APX; E'', intermediate formed from E'S by loss of superoxide; E_L, an APX species with modified specificity for reducing substrates (active only with ABTS and ascorbate).

Scheme 2 Simplified model of the mechanism of APX inactivation by H_2O_2

E', APX active site that has previously undergone reaction with H_2O_2 ; S, H_2O_2 ; E'S, complex between E' and S; E_i, inactive E; E_L, E with modified reducing substrate specificity (active only with ABTS and ascorbate).

pyrogallol assay, the curves obtained tended to approach 0%, but with ascorbate the curves tended to approx. 20% of the original activity (i.e. a fraction of APX seemed resistant to inactivation).

A plot of the natural logarithms of the percentage residual activities ($\ln A_R$) against time gave straight lines (Figure 1b) with slopes equivalent to the observed rate constants of inactivation (λ) [see Appendix, eqn. (A 17)]. The inactivation of APX by H_2O_2 clearly demonstrated saturation kinetics, as seen from the hyperbolic curves fitted to the plot of λ against $[\text{H}_2\text{O}_2]$ (Figure 1c). The dissociation constant (K_i), defined as the H_2O_2 concentration giving $\frac{1}{2}\lambda_{\max}$, was similar (means $\pm$ S.D.) with either the ascorbate ($0.80 \pm 0.12 \mu\text{M}$) or the pyrogallol ($0.86 \pm 0.10 \mu\text{M}$) assay and indicated a high affinity of APX for H_2O_2 . However, the maximum apparent rate constant of inactivation ($\lambda_{\max}$) [see Appendix, eqns. (A 18) and (A 21)] calculated from the curve fitted to each data set was different: $\lambda_{\max}$ for ascorbate was $(2.13 \pm 0.08) \times 10^{-4} \text{ s}^{-1}$, $\lambda_{\max}$ for pyrogallol was $(3.00 \pm 0.11) \times 10^{-4} \text{ s}^{-1}$. This variation in the apparent rate constants and, above all, the different final activity levels observed with different reducing substrates are consequences of the kinetics describing the proposed mechanism shown in Schemes 1 and 2.

When ascorbate or ABTS (or any other substrate that reveals a resistant fraction of APX; see below) is used to determine the enzyme activity [see Appendix, eqn. (A 21)], then:

$$\lambda_{\max} = k_i = (2.13 \pm 0.08) \times 10^{-4} \text{ s}^{-1}$$

Figure 2 Sensitivity to inactivation of APX at different molar ratios of H_2O_2

APX ($[E] = 0.3\text{--}0.5 \mu\text{M}$) was incubated with molar excesses of peroxide at the ratios indicated in phosphate buffer (50 mM, pH 7.0, 100 μl total volume). When the reaction was complete (16–18 h incubation) the percentage residual activities were measured with ascorbate (●) or pyrogallol (▲). Lines were fitted by using SigmaPlot for Windows and the intercepts at the x-axis were determined.

When pyrogallol or guaiacol (or any other substrate that does not reveal resistant APX) is used then [see Appendix, eqn. (A 18)]:

$$\lambda_{\max} = k_4 + k_i = (3.00 \pm 0.11) \times 10^{-4} \text{ s}^{-1}$$

thus:

$$k_4 = (0.87 \pm 0.19) \times 10^{-4} \text{ s}^{-1}$$

and because r , the partition ratio between the formation of the enzymic forms E_i and E_L (discussed below) is:

$$r = k_4/k_i$$

Therefore

$$r = 0.41 \pm 0.26$$

(Note that in this case r does not represent a partition between catalytic and inactivatory pathways as it does for HRP-C [12].)

Catalase activity of APX

The results of oxygraph experiments showed that APX did not possess any significant catalase-like activity. Active and heat-inactivated (boiled) APX gave similar traces. This result was consistent with the single-exponential time courses observed for the reaction and the low values of r obtained in incubation experiments below.

Number of equivalents of H_2O_2 required for inactivation of APX

APX was sensitive to inactivation by H_2O_2 , as demonstrated by values of 2–3 obtained for the intercepts at the x-axis in plots of residual activity (A_R) against the molar ratio of H_2O_2 to APX active sites (E), when A_R was measured with the different assays. Figure 2 shows the plots with the ascorbate and pyrogallol activity assays. When the residual activities were measured with ascorbate or ABTS the plots deviated from linearity at higher substrate-to-enzyme ratios. Activity was not fully lost even at

Figure 3 Protection of APX by ascorbate against inactivation by H₂O₂

APX ([E] = 0.5 μM) was incubated under the same conditions as specified in the legend to Figure 2, except that ascorbate was present in the samples at concentrations of 0 (●), 1.0 (■), 10.0 (▲) and 100.0 μM (▼). Results obtained with guaiacol instead of ascorbate were very similar.

[H₂O₂]-to-[E] ratios as high as 1000 (results not shown); a residual activity, measured with ascorbate, of 9.6% remained. When one of the phenolic substrates pyrogallol or guaiacol was used as the reducing substrate in the activity assays, this deviation from linearity was not observed and APX activity was decreased to immeasurably low levels by incubation with H₂O₂. When *m*-CPBA was substituted for H₂O₂ in the incubations, very similar results were obtained (results not shown).

From Scheme 1 and the stoichiometry of the reaction it can be seen that two molecules of H₂O₂ per APX active site are required to generate the inactive enzyme form, E_i, and three equivalents are required to make the enzyme form (with altered activity toward substrates), E_L [see Appendix, eqns. (A 1) and (A 2)]. Thus no enzyme turnover occurs but there is a partition ratio, *r*, obtained by the endpoint incubation method, which describes the formation of E_i and E_L with a value of *r* = 0.66 ± 0.15 [see Appendix, eqn. (A 15), and Figure 2], in good agreement with the value obtained from the kinetic constants above.

Protection of APX by reducing substrate against inactivation by H₂O₂

The presence of ascorbate or guaiacol in the incubation medium had a strong protective effect on the enzyme (Figure 3; results for ascorbate are shown). With 10 equiv. of H₂O₂, approx. 80% inactivation (20% residual activity) was seen in the absence of reducing substrate (with the use of the ascorbate activity assay), whereas the addition of 2 equiv. of reducing substrate lowered the inactivation to less than 20% (more than 80% residual activity); 90% residual activity was measured with 20 equiv. of reducing substrate, and 100% protection (no measurable inactivation) was afforded by the inclusion of 200 equiv. of reducing substrate.

Kinetic model

To account for these results, a kinetic model (Schemes 1 and 2 and Appendix) has been developed that uses as its basis a previous theoretical examination of the kinetics of mechanism-based inactivation (suicide inactivation) [32].

The principal characteristics of the mechanism are as follows. A species E' is formed by the reaction of an active site of APX (E) with H₂O₂. The identity of E' is not clear because APX compound I (apparent rate constant of formation at pH 7.8, *k*₁ = 8.0 × 10⁷ M⁻¹ s⁻¹ [25]) is unstable and undergoes conversion into another species with a rate constant, determined by stopped-flow, of *k*₂ = 0.8 ± 0.01 s⁻¹, this value being independent of H₂O₂ concentration in the range 0–8 μM (E. L. Raven and L. Lad, unpublished work). This enzyme form, when generated by incubation with 1 equiv. of H₂O₂ per APX active site, exhibits approx. 80% of the activity with reducing substrates of native APX. In the presence of additional H₂O₂, an enzyme–substrate complex, E'S, is made. There is a partition from complex E'S leading either directly to the inactive form, E_i, or, by the release of O₂^{•-}, to the intermediate E', which can react with a third equivalent of H₂O₂ to generate E_L. E_L possesses modified substrate specificity such that no reaction is observed with phenolics (e.g. pyrogallol, guaiacol), but approx. 20% of the original activity of APX remains with ascorbate and ABTS. This behaviour manifests itself as the difference in the λ_{max} observed in the time courses of inactivation by using different reducing substrates in the assays and also in the divergence from linearity in incubation experiments with ascorbate or ABTS (Figure 2).

Because no enzymic turnover of H₂O₂ occurs (i.e. there is no catalytic cycle) in the absence of reducing substrate but several molecules of H₂O₂ are required to inactivate each APX active site, the partition ratio between the routes to E_i and E_L is observed to have a value of *r* < 1.

DISCUSSION

In previous work we have examined the inactivation of the class III secretory plant peroxidase HRP [10–16]. HRP clearly undergoes mechanism-based inactivation with H₂O₂ and *m*-CPBA. Time dependence and saturation kinetics have been observed, along with protection of the enzyme by the presence of a reducing substrate. The stoichiometry of inactivator to enzyme has been closely defined with *m*-CPBA. Results from steady-state and rapid kinetics are consistent, and a detailed kinetically defined model of the inactivatory process has been developed [16]. In the present study we have examined the process of inactivation of a class I peroxidase, APX, and found that it shares similarities with, but also exhibits differences from, HRP.

The inactivation of APX by H₂O₂ was time dependent and the reaction showed saturation kinetics (Figure 1). The kinetically derived affinity of APX for H₂O₂ was much higher than that of HRP [*K*₁^{APX} = 0.8 μM, *K*₁^{HRP-C} = (1.3 ± 0.2) mM] [13,14]. APX was also much more sensitive to H₂O₂, as shown by the requirement of each active centre of APX for only approx. 2–3 molecules of H₂O₂ to be inactivated (Figure 2). This value was much lower than the equivalent value of 625 previously measured for the principal basic isoenzyme (form C) of HRP (*r* = 335 for non-glycosylated recombinant HRP-C) under the same conditions [13]. However, the cytosolic isoform of APX with which we performed this study was still considerably more resistant to inactivation than tAPX [27]. tAPX has been observed to undergo inactivation in medium containing less than 20 μM ascorbate and even as a result of the autoxidation of nanomolar concentrations of ascorbate (leading to the formation of H₂O₂) in dilute oxygenated solutions [9]. The sensitivity of tAPX might reflect the rather high level of ascorbate (up to 50 mM) normally found in the chloroplast [33], which protects the enzyme.

Despite APX's higher affinity for and greater sensitivity to H₂O₂, its inactivation proceeded an order of magnitude more slowly than that of HRP [*k*₁^{APX} = (2.13 ± 0.08) × 10⁻⁴ s⁻¹, *k*₁^{HRP-C}

$= (4.6 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$] [13,14]. This was a reflection of the low values of the kinetic constants controlling the inactivation pathway in APX.

Cytosolic APX was protected against inactivation by H_2O_2 by the presence of micromolar levels of the reducing substrates ascorbate or guaiacol in addition to H_2O_2 in the incubations (Figure 3). A similar effect has also been seen with HRP [10]. In both cases the reducing substrate protects the enzyme because peroxidase compound I preferentially oxidizes the reducing substrate rather than undergoing further reaction with H_2O_2 , which can lead to inactivation.

In incubations with the oxidizing substrate, *m*-CPBA, the behaviours of APX and HRP-C were much more alike. APX was as sensitive to inactivation by this peroxide as by H_2O_2 . The partition ratio for HRP-C with *m*-CPBA was only 2 [16].

The much greater sensitivity of HRP-C to inactivation by *m*-CPBA than by H_2O_2 has been attributed to the lack of the protective catalase-like (releasing oxygen gas) reaction that has been observed with H_2O_2 but that cannot occur with *m*-CPBA as the substrate [34,35]. The catalase-like reaction of H_2O_2 with HRP is the dominant pathway of enzyme turnover in the absence of reducing substrate and accounts for almost all the protection of HRP against mechanism-based inactivation by H_2O_2 . Oxygen experiments with APX confirmed that the enzyme did not possess a catalase-like cycle, as would be expected from its sensitivity to inactivation. It now seems that most peroxidases possess at least some catalase activity; they can be listed in the following order of activity: true catalases $\geq$ bacterial catalase-peroxidases $>$ chloroperoxidases $>$ HRP $>$ APX. There is presumably some reason why catalase-like activity has not evolved or has been lost in APX; this would suggest that APX's instability to peroxide might, in some way, be physiologically important.

The deviations from linearity of the plots of residual activity against the concentration ratios of APX with inactivator substrate at higher ratios (i.e. more substrate) when ascorbate and ABTS assays were used reveal that a fraction of the enzyme activity remained even at molar excesses of substrate up to 1000, despite the fact that most of the enzyme was inactivated with less than 10 equiv. of peroxide. Scheme 1 suggests that the explanation of this observation is that the enzyme does not complete a catalytic cycle (i.e. turnover) with H_2O_2 . APX forms either the inactive species (E_1) or the enzyme form with changed activity (E_L), which retains approx. 20% activity with substrates like ascorbate (resistant fraction) but does not demonstrate any reaction with phenolics. E_L therefore seems to be unable to withdraw electrons from phenolic substrates but can do so to a limited extent from acids such as ascorbate. There is evidence [36] that ascorbate and phenolic substrates, such as guaiacol and pyrogallol, bind to distinct electron transfer sites in APX. Specific modification of APX Cys-32, by either Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] or mutagenesis to serine, greatly decreased APX activity with ascorbate but did not appreciably affect phenolic oxidation. In contrast, the δ -meso-haem edge is believed to be the site of reaction for phenolics. Our results are consistent with the notion that there are two separate electron transfer sites in APX for phenolics and ascorbate, and that the phenolic site is blocked by inactivation.

Our studies so far have demonstrated that APX undergoes inactivation with H_2O_2 and also *m*-CPBA, as has been observed for HRP. The inactivatory process being observed can essentially be described as irreversible mechanism-based (suicide) inactivation, using the definition of Silverman [37]. However, with APX, the unusual situation arises where no enzyme turnover occurs and $r < 1$. Inactivation of APX therefore proceeds in much the same way as irreversible inhibition ($r = 0$) but each

active centre of APX requires several molecules of H_2O_2 for inactivation.

The structural changes that occur in the formation of the inactive enzyme, and the nature of this species, remain unclear. In preliminary UV/visible spectroscopic studies we have failed to identify a species similar to the inactive compound P670 verdohaemochrome seen in our previous work with HRP. However, the P670 of HRP is not particularly stable and easily decomposes; the equivalent species in APX might therefore be even less stable and harder to observe. P670 has also been observed to form in the reaction of wheat-germ peroxidase compound I, which seems to contain a protein radical, with excess H_2O_2 [23]. We did, however, notice a fall in Soret peak intensity, broadly in agreement with other studies [9] and suggesting that some haem degradation was occurring in APX. In addition, when excess ascorbate was added to the species that formed spontaneously from APX compound I (generated by the addition of 1 equiv. of H_2O_2 to the ferric enzyme), the spectrum of the original ferric species was not recovered. Similarly, the ferric spectrum was not fully recovered if the spontaneously formed species was allowed to evolve further over a period of several hours.

The sensitivity of APX to inactivation by its substrate H_2O_2 was surprising considering the enzyme's supposed role as a scavenger of this substance in cells as a protective measure against oxidative stress. Indeed, it has been shown in tobacco that, whereas APX functioned well under benign environmental conditions, under conditions of drought and bright illumination APX was completely inactivated, exposing the photosynthetic systems of the plants to severe damage. The introduction of the gene for bacterial catalase into tobacco plants allowed them to tolerate harsh environmental conditions much better than the wild-type plants [38]. In potato tubers stored at low temperature (causing oxidative stress due to the increase in dissolved O_2) APX did seem to protect the cells, at least until levels of ascorbate started to fall [39]. The same study also noted the protective effect of catalase, whose activity tended to increase as that of APX fell.

APX has been implicated in other cellular processes in plants in which its instability to H_2O_2 could be important. In response to pathogenic attack, plant cells undergo programmed cell death ('PCD'), termed the hypersensitive response (HR), to form a layer of dead cells as a barrier to the spread of disease. It is widely believed that reactive oxygen species such as H_2O_2 and $\text{O}_2^{\cdot -}$ are instrumental in mediating the HR ([40] and references therein); however, the action of APX could decrease their effectiveness. It has been shown that APX expression is suppressed at the post-transcriptional stage so that no new enzyme is produced during the HR [40]. The inactivation of APX by H_2O_2 at this stage could then further enhance the HR.

There is also evidence that H_2O_2 acts as a developmental signal in the differentiation of secondary walls in cotton fibres and other structures, such as tracheal elements, in other plants and that the scavenging of H_2O_2 can prevent such differentiation ([41] and references therein).

If we take into account observations such as those described above, it seems likely that the inactivation of APX by peroxide under certain circumstances could have important implications in plant development. The role of the different isoenzymes of APX with their specific localizations within plant cells is an area of plant biochemistry and physiology that still leaves many questions to be answered.

The purification of the APX used in this study was performed by Mr D. Turner and Mr K. Singh. This work was supported in part by a grant from the EU Training and Mobility of Researchers Programme – TMR Network, 'Peroxidases in Agriculture,

the Environment and Industry' (Contract FMRX-CT98-0200), and from Comisión Interministerial de Ciencia y Tecnología (Spain), CICYT-ALI98-0524. A. N. P. H. holds a fellowship from the EU TMR Network.

REFERENCES

- Welinder, K. G. (1992) Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Struct. Biol.* **2**, 388–393
- Welinder, K. G. (1991) Bacterial catalase–peroxidases are gene duplicated members of the plant peroxidase superfamily. *Biochim. Biophys. Acta* **1080**, 215–220
- Asada, K. (1992) Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. *Physiol. Plant.* **85**, 235–241
- Miyake, C. and Asada, K. (1992) Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. *Plant Cell Physiol.* **33**, 541–553
- Miyake, C., Cao, W. and Asada, K. (1993) Purification and molecular properties of thylakoid-bound ascorbate peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **34**, 881–889
- Nakano, Y. and Asada, K. (1987) Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant Cell Physiol.* **28**, 131–140
- Chen, G. and Asada, K. (1989) Ascorbate peroxidase in tea leaves: occurrence of two isoenzymes and the differences in their enzymatic and molecular properties. *Plant Cell Physiol.* **30**, 987–998
- Jespersen, H. M., Kjærsgård, I. V. H., Østergaard, L. and Welinder, K. G. (1997) From sequence analysis of three novel ascorbate peroxidases from *Arabidopsis thaliana* to structure, function and evolution of seven types of ascorbate peroxidase. *Biochem. J.* **326**, 305–310
- Miyake, C. and Asada, K. (1996) Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; hydrogen peroxide decomposes compound I of ascorbate peroxidase. *Plant Cell Physiol.* **37**, 423–430
- Arnao, M. B., Acosta, M., del Río, J. A. and García-Cánovas, F. (1990) Inactivation of peroxidase by hydrogen peroxide and its protection by a reductant substrate. *Biochim. Biophys. Acta* **1038**, 85–89
- Acosta, M., Arnao, M. B. and Hernández-Ruiz, J. (1993) Inactivation of peroxidase by hydroperoxides. In *Plant Peroxidases: Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C. and Greppin, H., eds.), pp. 201–205, University of Geneva, Geneva
- Arnao, M. B., Acosta, M., del Río, J. A., Varón, R. and García-Cánovas, F. (1990) A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide. *Biochim. Biophys. Acta* **1041**, 43–47
- Hiner, A. N. P., Hernández-Ruiz, J., García-Cánovas, F., Smith, A. T., Arnao, M. B. and Acosta, M. (1995) A comparative study of the inactivation of wild-type, recombinant and two mutant horseradish peroxidase isoenzymes c by hydrogen peroxide and *m*-chloroperoxybenzoic acid. *Eur. J. Biochem.* **234**, 506–512
- Hiner, A. N. P., Hernández-Ruiz, J., Arnao, M. B., García-Cánovas, F. and Acosta, M. (1996) A comparative study of the purity, enzyme activity, and inactivation by hydrogen peroxide of commercially available horseradish peroxidase isoenzymes a and c. *Biotechnol. Bioeng.* **50**, 655–662
- Arnao, M. B., García-Cánovas, F. and Acosta, M. (1996) Role of the reductant substrates on the inactivation of horseradish peroxidase by *m*-chloroperoxybenzoic acid. *Biochem. Mol. Biol. Int.* **39**, 97–107
- Rodríguez-López, J. N., Hernández-Ruiz, J., García-Cánovas, F., Thorneley, R. N. F., Acosta, M. and Arnao, M. B. (1997) The inactivation and catalytic pathways of horseradish peroxidase with *m*-chloroperoxybenzoic acid. *J. Biol. Chem.* **272**, 5469–5476
- Mittler, R. and Zilinskas, B. A. (1991) Molecular cloning and nucleotide sequence analysis of a cDNA encoding pea cytosolic ascorbate peroxidase. *FEBS Lett.* **289**, 257–259
- Patterson, W. R. and Poulos, T. L. (1995) Crystal structure of recombinant pea cytosolic ascorbate peroxidase. *Biochemistry* **34**, 4331–4341
- Goodin, D. B., Mauk, A. G. and Smith, M. (1987) The peroxide complex of yeast cytochrome *c* peroxidase contains two distinct radical species, neither of which resides at methionine 172 or tryptophan 51. *J. Biol. Chem.* **262**, 7719–7724
- Mauro, J. M., Fishel, L. A., Hazzard, J. T., Meyer, T. E., Tollin, G., Cusanovich, M. and Kraut, J. (1988) Tryptophan-191 → phenylalanine, a proximal-side mutation in yeast cytochrome *c* peroxidase that strongly affects the kinetics of ferrocytochrome *c* oxidation. *Biochemistry* **27**, 6243–6256
- Erman, J. E., Vitello, L. B., Mauro, J. M. and Kraut, J. (1989) Detection of an oxyferryl porphyrin π -cation-radical intermediate in the reaction between hydrogen peroxide and a mutant cytochrome *c* peroxidase. Evidence for tryptophan-191 involvement in the radical site of compound I. *Biochemistry* **28**, 7992–7995
- Dolphin, D., Forman, A., Borg, D. C., Fajer, J. and Felton, R. H. (1971) Compounds I of catalase and horseradish peroxidase: π -cation radicals. *Rec. Res. Dev. Agr. Food Chem.* **68**, 614–618
- Converso, D. A. and Fernández, M. E. (1998) Evidence for an unusual electronic structure of wheat germ peroxidase compound I. *Arch. Biochem. Biophys.* **357**, 22–26
- Dunford, H. B. and Stillman, J. S. (1976) On the function and mechanism of action of peroxidases. *Coord. Chem. Rev.* **19**, 187–251
- Márquez, L. A., Quitoriano, M., Zilinskas, B. A. and Dunford, H. B. (1996) Kinetic and spectral properties of pea cytosolic ascorbate peroxidase. *FEBS Lett.* **389**, 153–156
- Patterson, W. R., Poulos, T. L. and Goodin, D. B. (1995) Identification of a porphyrin π -cation radical in ascorbate peroxidase compound I. *Biochemistry* **34**, 4342–4345
- Amako, K., Chen, G. and Asada, K. (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant Cell Physiol.* **35**, 497–504
- Davies, D. M., Jones, P. and 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
- Pourchert, C. J. and Campbell, J. R. (1974) The Aldrich Library of NMR spectra, vol. 6, p. 146, Aldrich Chemical Company, Milwaukee, WI
- García-Cánovas, F., Tudela, J., Varón, R. and Vázquez, A. M. (1989) Experimental methods for kinetic study of suicide substrates. *J. Enz. Inhib.* **3**, 81–90
- Mittler, R. and Zilinskas, B. A. (1991) Purification and characterisation of pea cytosolic ascorbate peroxidase. *Plant Physiol.* **97**, 962–968
- Tudela, J., García-Cánovas, F., Varón, R., García-Carmona, F., Gálvez, J. and Lozano, J. A. (1987) Transient-phase kinetics of enzyme inactivation induced by suicide substrates. *Biochim. Biophys. Acta* **912**, 408–416
- Salin, M. L. (1987) Toxic oxygen species and protective systems in the chloroplast. *Physiol. Plant.* **72**, 681–689
- Arnao, M. B., Hernández-Ruiz, J., Varón, R., García-Cánovas, F. and Acosta, M. (1995) The inactivation of horseradish peroxidase by *m*-chloroperoxybenzoic acid, a xenobiotic hydroperoxide. *J. Molec. Cat. A: Chem.* **104**, 179–191
- Chamulitrat, W., Takahashi, N. and Mason, R. P. (1989) Peroxyl, alkoxyl, and carbon-centred radical formation from organic hydroperoxides by chloroperoxidase. *J. Biol. Chem.* **264**, 7889–7899
- Mandelman, D., Jamal, J. and Poulos, T. L. (1998) Identification of two electron-transfer sites in ascorbate peroxidase using chemical modification, enzyme kinetics, and crystallography. *Biochemistry* **37**, 17610–17617
- Silverman, R. B. (1995) Mechanism based enzyme inactivators. *Methods Enzymol.* **249**, 240–283
- Shikanai, T., Takeda, T., Yamauchi, H., Sano, S., Tomizawa, K.-I., Yokota, A. and Shigeoka, S. (1998) Inhibition of ascorbate peroxidase under oxidative stress in tobacco having bacterial catalase in chloroplasts. *FEBS Lett.* **428**, 47–51
- Mizuno, M., Kamei, M. and Tsuchida, H. (1998) Ascorbate peroxidase and catalase co-operate for protection against hydrogen peroxide generated in potato tubers during low-temperature storage. *Biochem. Molec. Biol. Int.* **44**, 717–726
- Mittler, R., Feng, X. and Cohen, M. (1998) Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco. *Plant Cell* **10**, 461–473
- Potikha, T. S., Collins, C. C., Johnson, D. I., Delmer, D. P. and Levine, A. (1999) The involvement of hydrogen peroxide in the differentiation of secondary wall in cotton fibers. *Plant Physiol.* **119**, 849–858

APPENDIX

The proposed mechanism for the reaction of APX with H_2O_2 leading to enzyme inactivation is shown in Scheme 1.

E represents the concentration of APX active sites other than the dimer.

The stoichiometry of the pathway leading to E_1 is:

and for the generation of E_L it is:

where the exact natures of E_1 and E_L have not been determined.

Experiments on the end-point residual activity

The equation for partition ratio (*r*) was developed as follows.

The balance of material in the mechanism in the case of the phenolic substrates where the residual activity falls to 0 is:

$$[S_0] = 2[E_i] + 3[E_L] \quad (\text{A } 3)$$

where $[S_0]$ is the initial $[H_2O_2]$.

The differential equations for the partition between E_i and E_L are:

$$d[E_L]/dt = k_4[E'S] \quad (\text{A } 4)$$

$$d[E_i]/dt = k_i[E'S] \quad (\text{A } 5)$$

Removing time from eqns. (A 4) and (A 5):

$$d[E_L]/d[E_i] = k_4/k_i \quad (\text{A } 6)$$

Integrating eqn. (A 6); $[E_L]_0 \rightarrow [E_L]$ and $[E_i]_0 \rightarrow [E_i]$:

$$[E_L] = [E_i]k_4/k_i \quad (\text{A } 7)$$

Substituting eqn. (A 7) into eqn (A 3):

$$[S_0] = 2[E_i] + 3([E_i]k_4/k_i) \quad (\text{A } 8)$$

Rearranging:

$$[S_0] = [E_i](2 + 3k_4/k_i) \quad (\text{A } 9)$$

and:

$$[E_i] = \frac{1}{2 + 3k_4/k_i} [S_0] \quad (\text{A } 10)$$

The balance of material with respect to E is:

$$[E_0] = [E_a] + [E_L] + [E_i] \quad (\text{A } 11)$$

where $[E_0]$ is the initial concentration of APX active sites (E) and $[E_a]$ is residual active E.

Substituting eqns. (A 7) and (A 10) into eqn. (A 11) we obtain:

$$[E_0] - [E_a] = \frac{1 + k_4/k_i}{2 + 3k_4/k_i} [S_0] \quad (\text{A } 12)$$

Dividing by $[E_0]$ and rearranging gives:

$$A_R = \frac{[E_a]}{[E_0]} = \frac{1 + k_4/k_i}{2 + 3k_4/k_i} \frac{[S_0]}{[E_0]} \quad (\text{A } 13)$$

$[E_a]$ can be determined from the remaining APX activity after incubation (A_i) and $[E_0]$ from the original APX activity (A_0). Thus both quantities on the left of eqn. (A 13) can be observed experimentally.

From the intercept at the x-axis of a plot of A_R against

$[S_0]/[E_0]$ (Figure 2) we see that, for pyrogallol and guaiacol, $A_R = 0$ when $[S_0]/[E_0] = 2.4$.

From Schemes 1 and 2 we define:

$$r = k_4/k_i \quad (\text{A } 14)$$

Thus, by using eqns. (A 13) and (A 14):

$$r = 0.66 \pm 0.15$$

which is in good agreement with the value obtained kinetically.

Kinetics of inactivation

Under conditions where $[S_0] \geq 15[E_0]$ the mechanism shown in Scheme 1 can be simplified to Scheme 2.

For substrates such as pyrogallol or guaiacol, for which no enzyme resistance is observed, $[E_L] \equiv [E_i]$.

The differential rate equation that describes the disappearance of the active enzyme, E_a , is:

$$-\frac{d[E_a]}{dt} = \frac{d[E_i]}{dt} = \frac{(k_4 + k_i)[E_a][S_0]}{K_i + [S_0]} \quad (\text{A } 15)$$

$$-\frac{d[E_a]}{dt} = \frac{(k_4 + k_i)[S_0]}{K_i + [S_0]} dt \quad (\text{A } 16)$$

Integrating between $[E_0]$ for $t = 0$ and $[E_a]$ for $t = t$ gives:

$$\ln \frac{[E_a]}{[E_0]} = -\frac{(k_4 + k_i)[S_0]}{K_i + [S_0]} t = -\lambda t \quad (\text{A } 17)$$

where λ is the observed rate constant of inactivation. Thus in a plot of λ against $[S_0] - [E_0]$ the maximum rate of inactivation ($\lambda_{\max}$) and the affinity of E' for S (K_i) can be obtained from a non-linear regression fit. In this case:

$$\lambda_{\max} = k_4 + k_i \quad (\text{A } 18)$$

Note that the generation of 1 mol of E' from E (Scheme 1) consumes 1 mol of S; therefore the plot and regression are done against $[S_0] - [E_0]$.

For ascorbate and ABTS, for which a resistant fraction of enzyme is detected, $[E_L] \neq [E_i]$.

The differential equations are:

$$-\frac{d[E_a]}{dt} = \frac{d[E_i]}{dt} = \frac{k_i[E_a][S_0]}{K_i + [S_0]} \quad (\text{A } 19)$$

yielding:

$$\ln([E_a]/[E_0]) = -\lambda' t = -k_i t \quad (\text{A } 20)$$

Therefore in a plot of λ' against $[S_0] - [E_0]$:

$$\lambda'_{\max} = k_i \quad (\text{A } 21)$$

Thus:

$$\lambda_{\max} - \lambda'_{\max} = k_4 \quad (\text{A } 22)$$

and additionally:

$$r = k_4/k_i \quad (\text{A } 23)$$

Received 17 January 2000/6 March 2000; accepted 24 March 2000