Plant Cell Physiol. 37(4): 423–430 (1996)

Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of Ascorbate; Hydrogen Peroxide Decomposes Compound I of Ascorbate Peroxidase

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One of the characteristic properties of ascorbate peroxidase (APX), which distinguishes it from guaiacol peroxidase, Cyt *c* peroxidase and glutathione peroxidase, is the rapid inactivation of the enzyme under conditions where an electron donor is absent. When thylakoid-bound APX (tAPX) in 100 μM ascorbate was diluted 500-fold with an ascorbate-depleted medium, the enzymatic activity was lost with half time of about 15 s. The inactivation of tAPX was suppressed under anaerobic conditions and also by the addition of catalase, but it was unaffected by the addition of superoxide dismutase. These observations suggest that hydrogen peroxide at nanomolar levels, produced by autooxidation of ascorbate at lower than micromolar levels, might participate in the inactivation of tAPX. The participation of hydrogen peroxide was confirmed by the inactivation of tAPX upon incubation with hydrogen peroxide under anaerobic conditions. In the absence of ascorbate, the heme of the two-electron-oxidized intermediate of tAPX (designated Compound I) is decomposed by hydrogen peroxide. Thus, the instability of Compound I to hydrogen peroxide is responsible for the inactivation of APX when ascorbate is not available for Compound I and the enzyme cannot turnover.

Key words: Ascorbate — Ascorbate peroxidase (EC 1.11.1.11) — Compound I — Hydrogen peroxide — Inactivation — Peroxidase.

Ascorbate peroxidase (APX) is an H_2O_2-scavenging peroxidase that uses ascorbate as an electron donor in plants and algae (Asada 1992). APX occurs in two isoforms with respect to its cellular localization. One isoform of APX is localized in chloroplasts and has been found in both a thylakoid-bound form (tAPX; Miyake and Asada 1992, Miyake et al. 1993) and a soluble form in the stroma (sAPX; Nakano and Asada 1987, Chen and Asada 1989). tAPX scavenges the H_2O_2 that is photoproduced in the thylakoids (Miyake and Asada 1992). The other isoform of APX is localized in the cytosol and its function seems to be the scavenging of the H_2O_2 produced in the cytosol. Furthermore, tAPX and sAPX are characterized by a higher specificity for ascorbate as an electron donor (Nakano and Asada 1987, Chen and Asada 1989, Miyake and Asada 1992) than that of cytosolic APX, which catalyzes the oxidation of phenols at higher rate than that of ascorbate (Chen and Asada 1989). The chloroplastic APXs have characteristic amino acid sequences in their amino-terminal regions (Chen et al. 1992b, Miyake et al. 1993), which differ from those of cAPX. The molecular mass of tAPX is also about 10 kDa greater than that of sAPX (Miyake et al. 1993).

APXs from higher plants are inhibited by thiol-modifying reagents, such as *p*-chloromercuribenzoate (pCMB; Nakano and Asada 1987, Chen and Asada 1989, Mittler and Zilinskas 1991a, Miyake and Asada 1992), while APX from *Euglena gracilis* z (Shigeoka et al. 1980), guaiacol peroxidases from plants and Cyt *c* peroxidase from yeast are not. pCMB does not affect the absorption spectrum of tAPX in the visible range, indicating little interaction of the functionnal thiol group with the heme (Miyake et al. 1993). The characteristic spectrum of Compound I of tAPX has not, however, been observed upon the addition of H_2O_2 in the presence of pCMB, suggesting the participation of a thiol group in the oxidation of the heme by H_2O_2 (Miyake et al. 1993).

APX isozymes from higher plants are inhibited by the thiyl radicals of GSH and dithiothreitol and by the phenoxy or aminoxy radicals of *p*-aminophenol, hydroxyurea and hydroxylamine (Chen and Asada 1990, 1992a, Miyake et al. 1993). These radicals are generated by H_2O_2 in a reaction catalyzed by APX at its reaction center. Thus, the enzyme is inactivated by a suicide mechanism. These radicals are also produced in reactions catalyzed with horseradish peroxidase (HRP), but they do not inhibit the activity of HRP, in this respect, guaiacol peroxidases such as HRP are also distinct from APX.

APX loses activity when the concentration of ascorbate is below 20 μM. This inactivation explains why APX was not found in early studies. The half-inactivation times of tAPX and sAPX are only 15 s, and that of cAPX is 40–60 min, if the concentration of ascorbate is below 2 μM (Nakano and Asada 1987, Chen and Asada 1989, Miyake and Asada 1992, Amako et al. 1994). This inactivation is suppressed by removal of dioxygen (Nakano and Asada

Abbreviations: APX, ascorbate peroxidase; cAPX, cytosolic APX; HRP, horseradish peroxidase; sAPX, stromal APX; tAPX, thylakoid-bound APX; pCMB, *p*-chloromercuribenzoate.

1987). Thus, it appears that an autooxidation product might inactivate APX. We have now found that tAPX is inactivated by H_2O_2 at nanomolar levels, even under anaerobic conditions, when the concentration of ascorbate is low. The rapid inactivation of tAPX can be explained by attack by H_2O_2 of the heme moiety of Compound I of tAPX. Part of this work has already been presented in a preliminary form (Miyake and Asada 1994a).

Materials and Methods

Enzymes—Thylakoid-bound ascorbate peroxidase (tAPX) was purified to homogeneity from spinach thylakoids as described by Miyake et al. (1993), and it was stored at −80°C in 50 mM potassium phosphate (pH 7.5)/0.1 mM EDTA/1 mM phenylmethyl sulfonate/5 mM ascorbate/1% (w/v) 3-[(3-chloramidopropyl)dimethyl-ammonio]-2-hydroxy-propanesulfonate. The concentration of tAPX was determined from the absorbance at 403 nm on the assumption of an absorption coefficient of 110 mM^{-1} cm^{-1} (Miyake et al. 1993). HRP (grade I-C) was obtained from Toyobo (Osaka, JAPAN). Its RZ, defined as $A_{403\,nm}/A_{275\,nm}$, was above 3.0, and its concentration was estimated on the assumption of an absorption coefficient of 100 mM^{-1} cm^{-1} at 403 nm (Nakajima and Yamazaki 1979).

Assay of APX—The activity of APX was determined as described previously (Nakano and Asada 1981) in a reaction mixture (1 ml) that contained 50 mM potassium phosphate (pH 7.0)/0.1 mM H_2O_2/0.5 mM ascorbate. No detergent was added to the reaction mixture because tAPX was not inactivated during measurements of the initial rate of the tAPX-catalyzed reaction under the present conditions. The H_2O_2-dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm assuming an absorption coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX was defined as the amount of enzyme that oxidized 1 μmol of ascorbate per min at room temperature under the conditions of the reaction.

Results and Discussion

Inactivation of tAPX by dilution with ascorbate-depleted medium—One of the characteristic properties of APX is its rapid inactivation in ascorbate-depleted medium (Hossain and Asada 1984, Nakano and Asada 1987, Chen and Asada 1989). This property is also exhibited by tAPX purified from spinach thylakoids (Miyake and Asada 1992). When 1 nM tAPX in 100 μM ascorbate-containing medium was diluted 500-fold with mediun that contained 0.2–100 μM ascorbate, its activity decreased with time and with decreases in the concentration of ascorbate in the dilution medium (Fig. 1A). The inactivation by such dilution was not, however, observed with higher concentrations of ascorbate in the dilution medium. Little inactivation was observed when dilution was performed with medium that contained ascorbate at concentrations higher than 30 μM (Fig. 1B).

When the solutions of the enzyme was diluted under anaerobic conditions, no inactivation of tAPX was observed (Fig. 2). This result was consistent with the results for sAPX from spinach (Nakano and Asada 1987) and cAPX from tea (Amako et al. 1994) and it suggests the participation of an autooxidation product in the inactivation of tAPX. The effects of catalase and superoxide dismutase

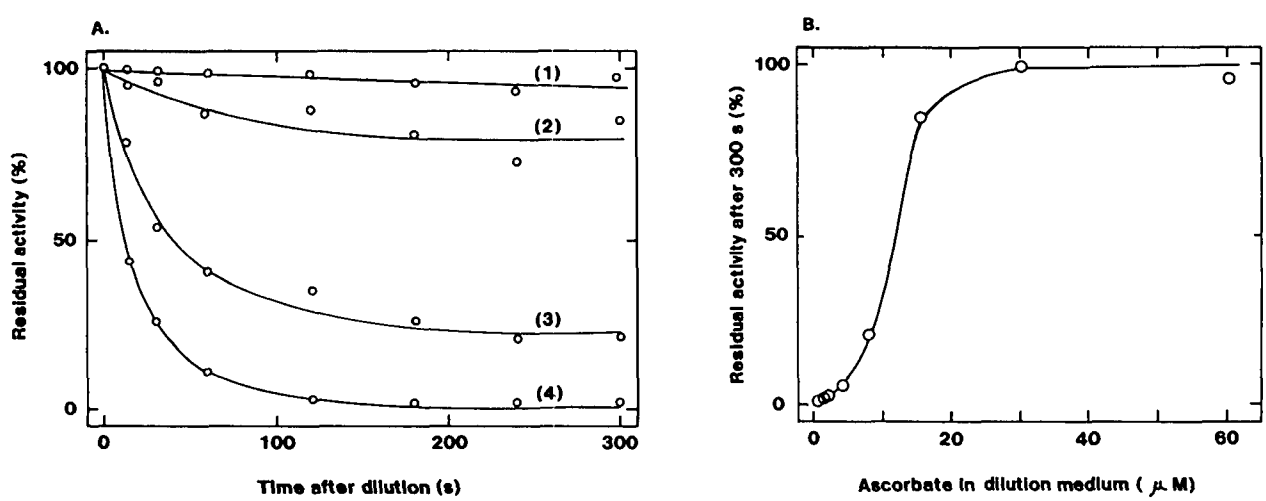

Fig. 1 Inactivation of tAPX purified from spinach thylakoids by 500-fold dilution with ascorbate-depleted and ascorbate-containing media. (A) A solution of tAPX (1 pmol in 2 μl of 100 μM ascorbate/50 mM potassium phosphate, pH 7.0) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench inactivation of tAPX, and the tAPX activity was assayed after the addition of 0.1 mM H_2O_2, as described in Materials and Methods. Where indicated, dilution of tAPX was performed with a solution that contained 0.2–100 μM ascorbate [(1) 100 μM; (2) 16.0 μM; (3) 8.0 μM; (4) 0.2 μM]. Activity of tAPX in the presence of ascorbate at 0 min was 0.06 units and was taken as 100%. (B) Residual activity of tAPX 300 s after dilution with 50 mM potassium phosphate buffer, pH 7.0, that contained ascorbate at the indicated concentrations. Dilution and the assay of tAPX activity after the dilution were performed in (A).

on the inactivation of tAPX by dilution with ascorbate-depleted medium were examined to determine whether tAPX is inactivated by either superoxide or H_2O_2, which would be produced via autooxidation. The inactivation of tAPX was suppressed by 80% by 1,000 units of catalase, but superoxide dismutase has no effect (Fig. 2). Thus, the H_2O_2 produced via autooxidation upon dilution of tAPX must have inactivated the enzyme.

The most likely candidate for the autooxidizable compound is the ascorbate in the stock solution of tAPX. In fact, when we diluted tAPX with ascorbate-depleted medium at various pH values, we found that the inactivation of tAPX was accelerated at higher pH values. The apparent half-times for inactivation of tAPX were 35 s at pH 6.5, 15 s at pH 7.0, and 4 s at pH 8.1 (Fig. 3). The pKa_1 and pKa_2 of ascorbate are 4.0 and 11.3, respectively (Njus and Kelly 1993), and the redox potentials between the monodehydroascorbate radical and the monoanionic form of ascorbate and between the radical and the dianionic form of ascorbate are 0.766 V and 0.076 V, respectively (Sapper et al. 1982). Therefore, the rate of autooxidation of ascorbate is high at high pH (Scarpa et al. 1983), with the resultant increased production of H_2O_2. If the inactivation rate of tAPX was unaffected by pH over the range of pH values examined, the high rate of inactivation rate of tAPX at high pH values would suggest the participation of the H_2O_2 produced via autooxidation of ascorbate in the inactivation. Furthermore, when the ascorbate in the stock solution of tAPX was removed by gel filtration on a Sephadex G-25 column (PD-10; Pharmacia, Uppsala, Sweden) no inactivation of tAPX was observed even under aerobic conditions (data not shown). This observation supports the proposed participation of the H_2O_2 produced by autooxidation of ascorbate in the inactivation of tAPX.

Inactivation of tAPX by addition of exogenous hydrogen peroxide under anaerobic conditions—In order to demonstrate directly the participation of H_2O_2 in the inactivation of tAPX, H_2O_2 was added to a solution of tAPX under anaerobic conditions. Under such anaerobic conditions, no inactivation was observed as a consequence of dilution (Fig. 2). tAPX (0.85 nM) was inactivated by H_2O_2 at nanomolar levels, and the rate of inactivation increased with increases in the concentration of H_2O_2 (Fig. 4). These results indicate that tAPX is actually inactivated by H_2O_2 if the concentration of the electron donor for tAPX, ascorbate, is below the micromolar range. Thus, the inactivation of tAPX by dilution was caused by the H_2O_2, at nanomolar levels, produced via autooxidation of micromolar levels of ascorbate that was derived from the stock solution of tAPX. Therefore we studied the inactivation mechanism of tAPX by H_2O_2 when the ascorbate concentration was low.

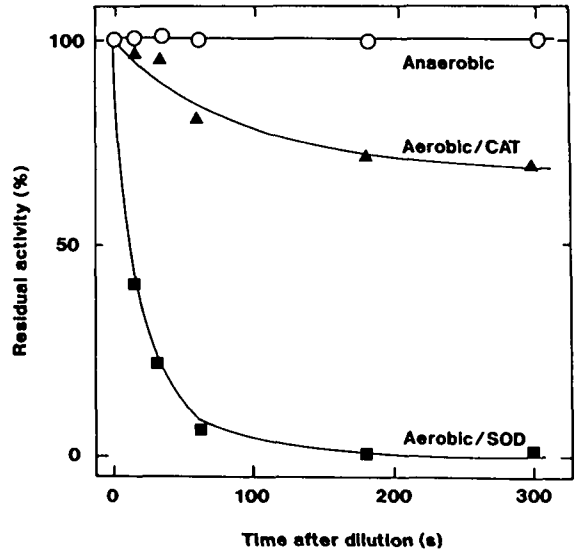

Fig. 2 Effects of dioxygen, catalase and superoxide dismutase on inactivation of tAPX by dilution with ascorbate-depleted medium. A solution of tAPX (1 pmol in 2 μl of 1 mM ascorbate/50 mM potassium phosphate, pH 7.0) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench the inactivation, and APX activity was assayed as described in the legend to Fig. 1. Aerobic/SOD, The dilution was performed with the buffer that contained CuZn-superoxide dismutase (200 units); Aerobic/CAT, the buffer contained catalase (1,000 units); Anaerobic, the buffer was rendered anaerobic by bubbling with argon gas and the dilution was performed under anaerobic conditions. Activity of tAPX in the presence of ascorbate at 0 min was 0.04 units and was taken as 100%.

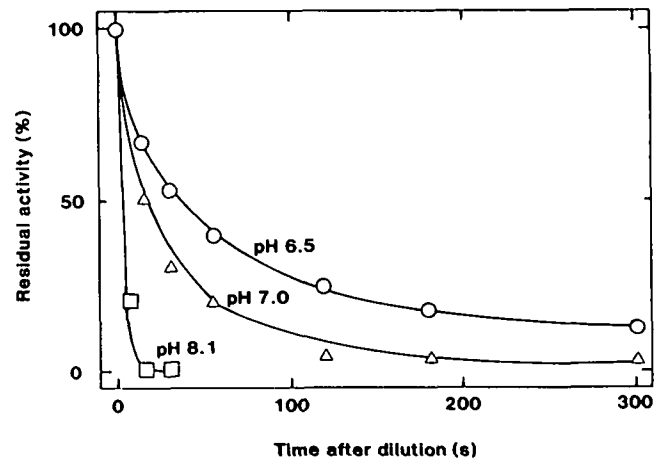

Fig. 3 Effects of pH on the inactivation of tAPX by dilution with ascorbate-depleted medium. A solution of tAPX (1 pmol in 2 μl of 1 mM ascorbate/50 mM potassium phosphate, pH 7.0) was diluted with 1 ml of 50 mM potassium phosphate buffer at the indicated pH (circles, pH 6.5; triangles, pH 7.0; squares, pH 8.1). At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench the inactivation of tAPX, and the tAPX activity was assayed as described in the legend to Fig. 1.

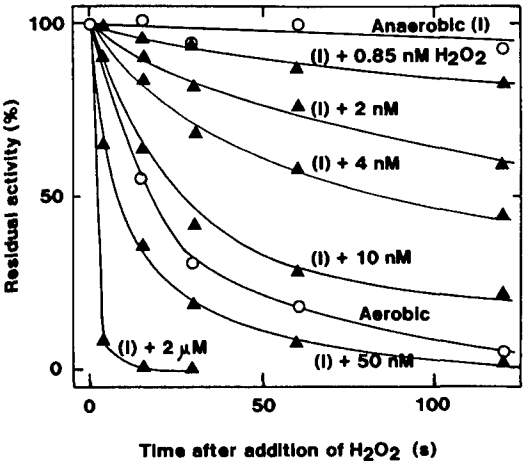

Fig. 4 Inactivation of tAPX by hydrogen peroxide in ascorbate-depleted medium under anaerobic conditions. A solution of tAPX (0.85 pmol in 2 μl of 1 mM ascorbate/50 mM potassium phosphate buffer, pH 7.0) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0, that contained H_2O_2 at the indicated concentrations under anaerobic conditions, unless otherwise specified. The final concentration of tAPX was 0.85 nM. At the indicated times, the inactivation was quenched by the addition of 0.5 mM ascorbate to the incubation mixture. Subsequently, APX activity was determined as described in the legend to Fig. 1. The activity of tAPX in the presence of ascorbate at 0 min was 0.034 units and was taken as 100%.

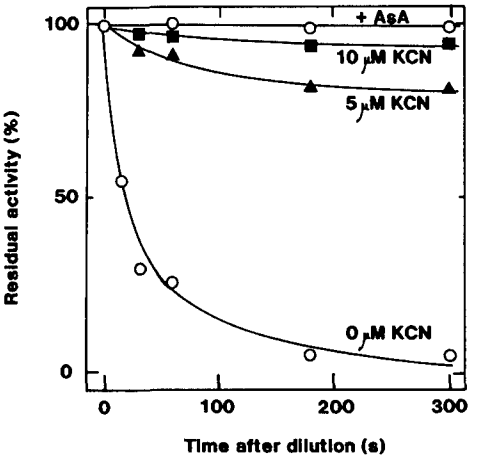

Fig. 5 Suppression by cyanide of the inactivation of tAPX by dilution with ascorbate-depleted medium. A solution of tAPX (1 pmol in 2 μl of 1 mM ascorbate/50 mM potassium phosphate, pH 7.0) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench the inactivation, and APX activity was assayed as described in the legend to Fig. 1. Where indicated, the dilution was performed with buffer that contained 0.5 mM ascorbate (AsA) or KCN at the indicated concentration. The activity of tAPX at 0 min in the absence of cyanide was 0.04 units and was taken as 100%. In the presence of 5 and 10 μM cyanide, the activities of tAPX at 0 min were 60% and 24% of that in the absence of cyanide, respectively, and these activities were taken as 100% for each respective treatment.

Hydrogen peroxide inactivates tAPX through the interaction with its heme moiety—tAPX participates in the following catalytic cycle (Miyake et al. 1993), designated the "peroxidase-ping pong" cycle (Dunford 1991).

$$APX(Fe^{III})R + H_2O_2 \xrightarrow{k_1} APX(Fe^{IV}=O)R\cdot + H_2O$$
Compound I
$$(k_1 = 1.2 \times 10^7\ M^{-1}\ s^{-1})$$

$$APX(Fe^{IV}=O)R\cdot + AsA \xrightarrow{k_2} APX(Fe^{IV}=O)R + MDA$$
Compound II
$$(k_2 \geq 10 \times k_3)$$

$$APX(Fe^{IV}=O)R + AsA \xrightarrow{k_3} APX(Fe^{III})R + H_2O + MDA$$
$$(k_3 = 2.1 \times 10^6\ M^{-1}\ s^{-1})$$

APX first reacts with H_2O_2 and is converted to the two-electron-oxidized intermediate, Compound I, in which the heme moiety is oxidized to the oxyferryl ($Fe^{IV}=O$) species and an organic group, R, either the porphyrin or the side chain of an amino acid, is oxidized to a free radical, R·. Compound I is reduced back to the resting ferric (Fe^{III}) state by two succesive one-electron reactions with ascorbate (AsA), producing two molecules of the one-electron-oxidized product of ascorbate, the monodehydroascorbate radical (MDA). The reaction rate constants (k_1, k_2 and k_3) are those that were previously determined for tAPX (Miyake et al. 1993).

To identify the site in APX that interacts with H_2O_2 to bring about the subsequent inactivation, the solution of enzyme was diluted with cyanide-containing ascorbate-depleted medium. Under such conditions, tAPX forms a cyanide complex upon dilution, and the heme moiety of tAPX cannot be oxidized to Compound I by the H_2O_2 that is produced via autooxidation of ascorbate. The inactivation of tAPX by dilution was suppressed when cyanide was included in the dilution buffer and the effect was marked when the concentration of cyanide was high (Fig. 5), suggesting that H_2O_2 does not inactivate tAPX by interaction with any sites other than the heme of tAPX. Thus, H_2O_2 seems to inactivate tAPX through an interaction with the heme of tAPX. In the "peroxidase-ping pong" cycle of tAPX, H_2O_2 rapidly oxidizes the heme of tAPX, and Compound I is formed. Therefore, we next studied the interaction of H_2O_2 with Compound I of tAPX.

Degradation of Compound I of tAPX by hydrogen peroxide—Native tAPX has an absorption spectrum characteristic of a ferric (Fe^{III}) high-spin state, with a Soret peak at 403 nm. On addition of an equimolar amount of H_2O_2 to a solution of native tAPX, the Soret peak was shifted to 415 nm (Fig. 6A), as observed in the case of Cyt *c* peroxidase (Yonetani and Anni 1987). The H_2O_2-oxidized

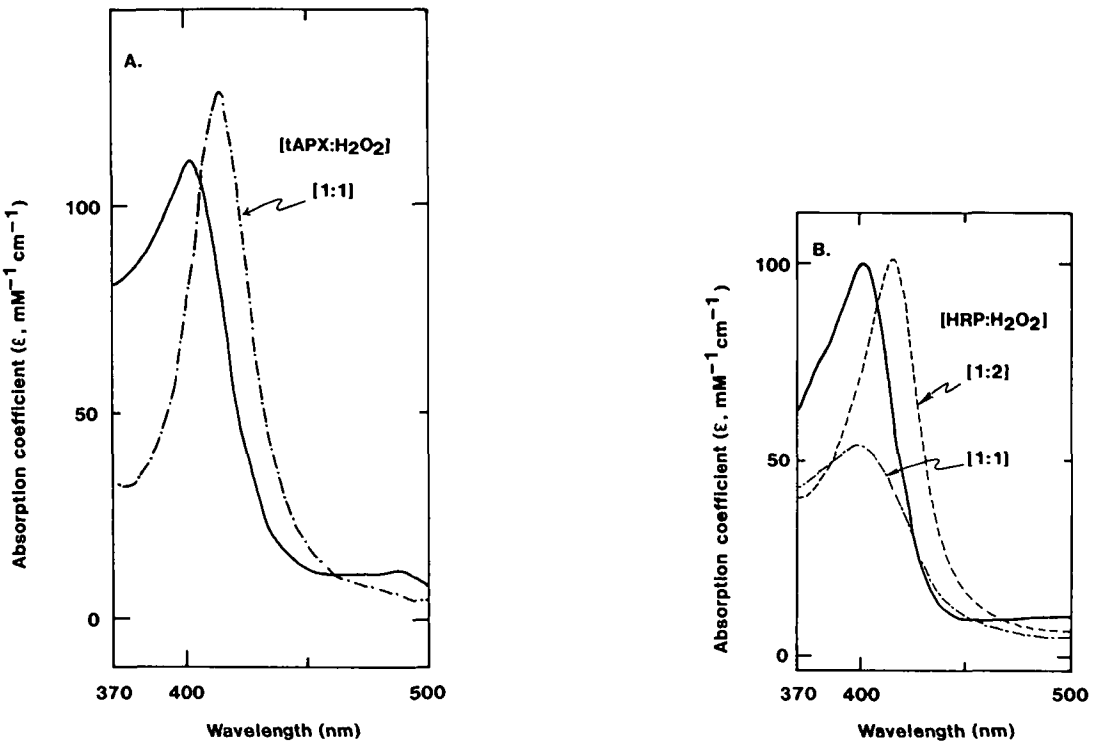

Fig. 6 Absorption spectra of tAPX (A) and HRP (B) and of their hydrogen peroxide-oxidized intermediates. (A) The absorption spectrum of a 4 μM solution of native tAPX was recorded in 50 mM potassium phosphate, pH 7.0, and 1% 3-[(3-chloramidopropyl)dimethyl-ammonio]-2-hydroxy-propanesulfonate (—), after removal of ascorbate from the stock solution of tAPX by gel-filtration chromatography on column of Sephadex G-25. Under these conditions no inactivation of tAPX was observed. Subsequently, 4.0 μM H_2O_2 (– · –) was added and the absorption spectrum of the H_2O_2-oxidized intermediate (Compound I) was recorded. (B) The absorption spectrum of a 4 μM solution of native HRP was recorded in 50 mM potassium phosphate, pH 7.0 (—). The spectra after addition of 4 μM (– · –) and 8 μM (----) H_2O_2 represent Compounds I and II, respectively (George 1953, Arnao et al. 1990). The absorption spectra were recorded at a scanning rate of 7.1 nm s^{-1}.

intermediate of Cyt *c* peroxidase has been proposed to correspond to heme-Fe^{IV}=O with a Trp radical (Sivaraja et al. 1989). In the case of HRP, the H_2O_2-oxidized intermediate (Compound I) has been proposed to correspond to heme-Fe^{IV}=O with a porphyrin π-cation radical (Dolphin et al. 1971). For comparison, the spectrum of the green Compound I of HRP is shown (Fig. 6B). However, on addition of H_2O_2 to tAPX no green color was detected. Thus, the H_2O_2-oxidized intermediate of tAPX seems to correspond to heme-Fe^{IV}=O with an amino acid residue radical. Moreover, a Trp residue is conserved between Cyt *c* peroxidase and APX (Mittler and Zilinskas 1991b, Kubo et al. 1992, Amako et al. unpublished). In contrast to Compound I of tAPX, it was reported recently that Compound I of cytosolic APX is green in color, with heme-Fe^{IV}=O and a porphyrin π-cation radical, resembling Compound I of HRP (Patterson et al. 1995).

Compound I of tAPX, formed upon the addition of an equimolar amount of H_2O_2, was stable at least for 20 min (Fig. 7A, 8). However, when more H_2O_2 was added to the solution of Compound I, the size of the Soret peak decreased with time, indicating the degradation of the heme moiety (Fig. 7B, C, 8). The rate of degradation of the heme of Compound I increased as the concentration of H_2O_2 was increased, as estimated from the decrease in absorbance at 415 nm after the addition of H_2O_2 (Fig. 8). An apparent rate constant for the degradation of the Compound I-heme by H_2O_2 (k_{deg}) was estimated on the assumption that the reaction follows second-order kinetics. A reciprocal plot of the decrease in the absorbance at 415 nm against time after the addition of an equimolar amount of H_2O_2 to Compound I (Fig. 8), yielded a straight line with a slope that gave a k_{deg} of 8×10^3 M^{-1} s^{-1}. In the same way, the rate constant for the inactivation of tAPX by H_2O_2 (k_{inac}) was estimated to be 7×10^5 M^{-1} s^{-1}, using the results (Fig. 4) observed on addition of 2 nM H_2O_2. These conditions correspond to the conditions where the ratio of concentrations of Compound I of tAPX to H_2O_2 was 1.2 to 1. The difference in values between k_{inac} and k_{deg} suggests that the degradation of the heme of Compound I of tAPX by H_2O_2 was followed by the inactivation of tAPX as a consequence of the interaction of Compound I with H_2O_2. An active site of

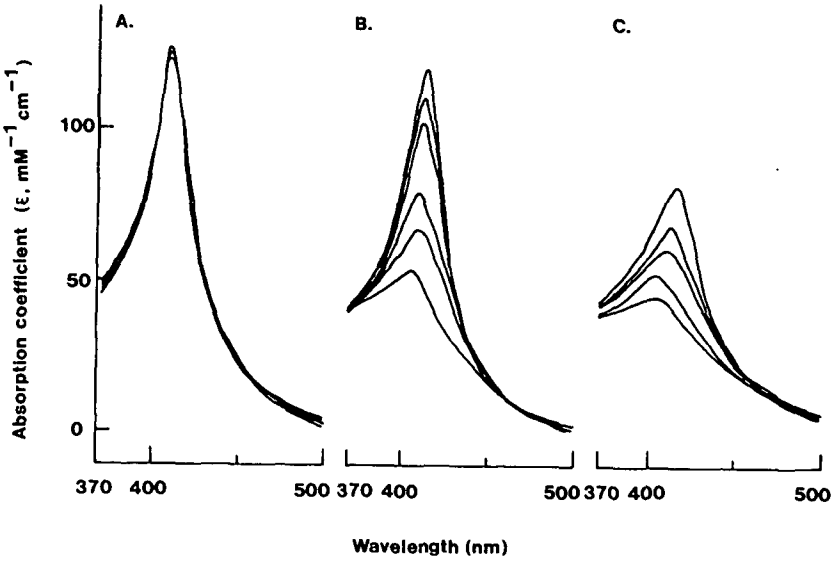

Fig. 7 Absorption spectrum of Compound I of tAPX and degradation of the Compound I by hydrogen peroxide. A 4 μM solution of native tAPX in 50 mM potassium phosphate, pH 7.0, and 1% 3-[(3-chloramidopropyl)dimethyl-ammonio]-2-hydroxy-propanesulfonate, was supplemented with 4 μM (A), 8 μM (B) or 80 μM (C) H_2O_2. The scanning rate was 7.1 nm s^{-1}. The peak of Compound I of tAPX was at 415 nm. The first spectrum was recorded 10 s after addition of H_2O_2 and then the recordings were repeated every 23 s, as shown from top to bottom.

tAPX other than the heme might have been modified by H_2O_2 and then the degradation of the heme by H_2O_2 might have been facilitated in the modified enzyme. Compound I of HRP was stable in the absence of an electron donor, resembling Compound I of APX (Fig. 6B). Unlike the case with tAPX, however, on addition of double the equimolar amount of H_2O_2 to HRP, Compound II with a peak at 413 nm appeared, via Compound I (Fig. 6B), consistent with the results of George (1953) and Arnao et al. (1990). Compound II of HRP also was stable (data not shown). Thus, the rapid decomposition of Compound I by H_2O_2 seems to be a characteristic property of tAPX.

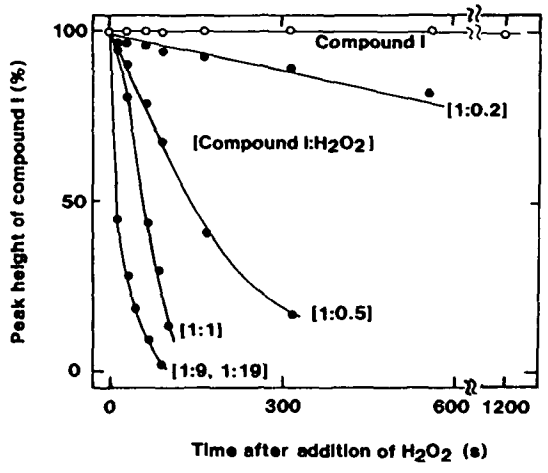

Fig. 8 Time courses of the decrease in the height of the peak of Compound I of tAPX. Addition of an equimolar amount of H_2O_2 to a 4 μM solution of native tAPX yielded Compound I, as in Fig. 6, and the peak height was taken as 100%. Subsequently, H_2O_2 was added to Compound I at the indicated molar ratios, and decreases in absorbance at 415 nm were recorded (closed circles). Open circles show the stability of Compound I of tAPX over the course of 20 min in the absence of H_2O_2.

Concluding remarks—The APXs from chloroplasts, namely, tAPX and sAPX, are rapidly inactivated when the concentration of ascorbate is in the micromolar range. We have shown here the inactivation of tAPX is caused by the H_2O_2, at nanomolar levels, that is produced by the autooxidation of ascorbate at micromolar levels. Furthermore, we have provided evidence that H_2O_2 inactivates the H_2O_2-oxidized intermediate (Compound I) of tAPX, with the decomposition of the heme moiety, when the concentration of ascorbate is too low for the operation of the catalytic cycle of the peroxidase. The apparent "K_m" for tAPX is 500 μM (Miyake et al. 1993). Thus, ascorbate at micromolar levels cannot reduce either Compound I or Compound II in the peroxidase ping-pong catalytic cycle. In figure, we propose a mechanism for the inactivation of tAPX by H_2O_2 when the concentration of ascorbate is far below its K_m value (Fig. 9).

At present, we do not know which site in Compound I of tAPX is primarily modified by H_2O_2 to inactivate the enzyme, and the subsequent degradation product of the heme moiety also is unknown. In the case of HRP, the interaction of Compound I with H_2O_2 allows formation of Compound II, with production of superoxide anion radicals, but for tAPX no such reaction was observed and the heme

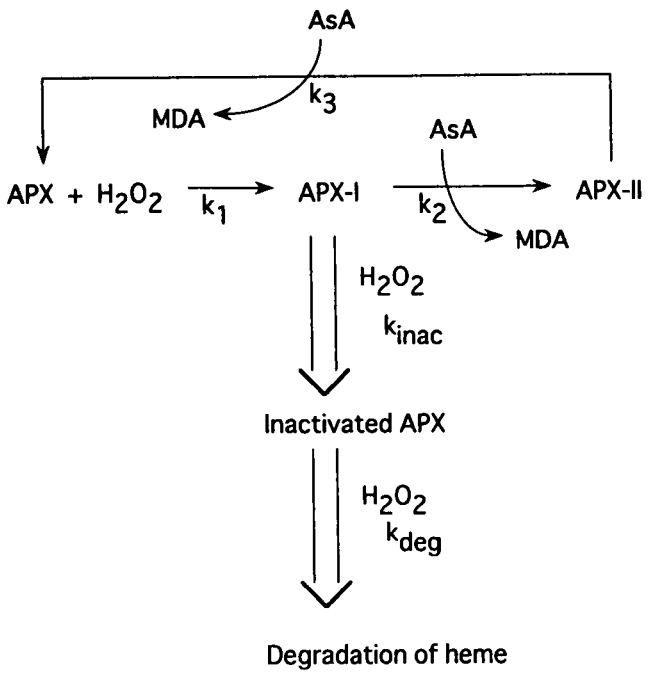

Fig. 9 Catalytic cycle of tAPX and inactivation of tAPX by hydrogen peroxide at low concentrations of ascorbate. APX-I and APX-II represent Compound I and Compound II of APX, respectively. AsA, Ascorbate; MDA, monodehydroascorbate. $k_1 = 1.2 \times 10^7\ M^{-1}\ s^{-1}$, $k_2 \geq 10 \times k_3$ and $k_3 = 2.1 \times 10^6\ M^{-1}\ s^{-1}$ (Miyake et al. 1993). $k_{inac} = 7 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{deg} = 8 \times 10^3\ M^{-1}\ s^{-1}$ (present work).

was decomposed (Fig. 6). Even though APX shows a high degree of homology in terms of amino acid sequence to Cyt *c* peroxidase (Chen et al. 1992b, Kubo et al. 1992, Mittler and Zilinskas 1991b, Amako et al. unpublished) and the tertiary structure and the microenvironment around heme of cAPX resemble those of Cyt *c* peroxidase (Patterson et al. 1995), Compound I of Cyt *c* peroxidase is stable against H_2O_2 (Yonetani 1965).

In chloroplasts of higher plants, ascorbate is formed at concentrations from 10 to 50 mM (Gerhardt 1964, Foyer et al. 1983). When superoxide is produced by the photoreduction of dioxygen in PSI and H_2O_2 is produced via disproportionation of the superoxide, in a reaction catalyzed by superoxide dismutase which is bound or attached to the thylakoid membrane (Hayakawa et al. 1985, Ogawa et al. 1995), the H_2O_2 is reduced to water by ascorbate in reactions catalyzed by tAPX and sAPX. The primary oxidation product of ascorbate in the APX-catalyzed reaction, the monodehydroascorbate radical, is rapidly reduced ($\approx 10^7\ M^{-1}\ s^{-1}$) to ascorbate by ferredoxin that is photoreduced in PSI (Miyake and Asada 1994b), and by NADH in a reaction catalyzed by monodehydroascorbate reductase (Sano et al. 1995). The reaction rate constants for superoxide dismutase and superoxide ($2 \times 10^9\ M^{-1}\ s^{-1}$), for APX and H_2O_2 ($10^7\ M^{-1}\ s^{-1}$) and for monodehydroascorbate radical and reduced ferredoxin ($10^7\ M^{-1}\ s^{-1}$), and the local concentrations of superoxide dismutase (1 mM), APX (1 mM) and ferredoxin (3 mM) in the vicinity of the PSI complex of thylakoids allow us to estimate the pseudofirst-order rate constant for each reaction. The half-times of the superoxide radical, H_2O_2 and the monodehydroascorbate radical are estimated to be 4×10^{-7} s, 7×10^{-5} s and 2×10^{-5} s, respectively. By contrast, the half time for photoproduction of superoxide in PSI is more than 5×10^{-2} s. Thus, the concentration of ascorbate remains far higher than that of H_2O_2 in chloroplasts. Therefore, the inactivation of tAPX by H_2O_2 is unlikely to occur in chloroplast. However, the inactivation of APX by the present mechanism is likely to occur under photooxidative stress. For example, when paraquat is applied, all of the electrons photogenerated in PSI are transferred to paraquat to produce its cationic radicals. The paraquat radicals are rapidly oxidized by dioxygen producing superoxide and then H_2O_2, and no photoreducing equivalents are available for the regeneration of ascorbate from monodehydroascorbate radicals. Under such conditions, the concentration of ascorbate gradually decreases within two min (Nakano and Asada 1980), and the APX would be inactivated as a result of the unavailability of ascorbate to Compound I.

The present work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (no. 04273101) from the Ministry of Education, Science and Culture of Japan and by a grant from the Human Frontier Science Program.

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(Received October 16, 1995; Accepted February 21, 1996)