Salicylic Acid Is a Reducing Substrate and Not an Effective Inhibitor of Ascorbate Peroxidase*

(Received for publication, June 9, 1997, and in revised form, June 25, 1997)

Mamuka Kvaratskhelia, Simon J. George, and Roger N. F. Thorneley
From the Nitrogen Fixation Laboratory, John Innes Centre, Norwich, NR4 7UH, United Kingdom

This communication describes the interactions of salicylic acid (SA) with plant ascorbate peroxidase (APX). Contrary to a recent report (Burner, J., and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11312–11316) we show conclusively that ascorbate oxidation by APX is not inhibited by SA (10 mM), but that SA is a slow reducing substrate of this enzyme. The suggestion that SA-dependent inhibition of APX in planta may result in the elevation of H₂O₂ levels, which in turn acts as a second messenger in systemic acquired resistance signaling, is therefore not tenable. We conclude that APX remains a key antioxidant during systemic acquired resistance following pathogenic infection of plants. The transient products of SA oxidation by APX appear to be SA free radicals that undergo subsequent chemistry. APX-dependent oxidation of SA could be essential for diminishing the detrimental effects of this phenolic acid on plant cells.

Salicylic acid (2-hydroxybenzoic acid) is believed to play an important role in plant defense responses against pathogen attack (see Refs. 1 and 2 and references therein). Many studies implicate SA as an essential signal in the development of the systemic acquired resistance (SAR) in several plant species. SAR is an inducible, long lasting, broad-based pathogen defense mechanism following infection of plants, particularly by a necrotizing pathogen. An understanding of the molecular basis of SAR may well lead to the development of both genetically engineered crops with enhanced disease resistance and novel chemicals that stimulate the plants' resistance to pathogens.

Although induction of SAR is associated with a significant increase in endogenous SA levels in plants (3, 4), the biochemical basis of this phenomenon is obscure. SA accumulation is essential for pathogenesis-related (PR) gene expression (5, 7). Exogenous application of SA to Arabidopsis or tobacco switches on the same set of SAR genes as pathogen infection and induces protection against the same spectrum of pathogens (5, 8). Experiments with transgenic plants that express a bacterial salicylate hydroxylase gene have shown that SA could be a transmissible signal (9). The constitutive expression of salicylate hydroxylase, encoded by the nahG gene from Pseudomonas putida, prevented the accumulation of SA in transgenic tobacco by converting it to catechol, which is inactive in SAR. Treatment of NahG plants with tobacco mosaic virus did not induce SAR (9) or SAR gene expression in systemic leaves (10). Grafting experiments involving NahG and wild-type tobacco have suggested that although SA is probably not a long distance signal, its presence in systemic tissue is absolutely required for PR protein expression and establishment of SAR (10). PR-1a gene expression in infected tissues is dependent on SA accumulation and supports the suggestion that at least local induction of this PR protein operates via salicylate signaling (11).

Klessig and co-workers (12–14) have suggested that one mechanism of SA action is to inhibit catalase and ascorbate peroxidase (APX) (15), thereby elevating the endogenous levels of H₂O₂ that result from either the oxidative burst associated with the hypersensitive response following pathogen attack or from metabolic processes (e.g., photorespiration, photosynthesis, and oxidative phosphorylation). According to this hypothesis, the elevated level of H₂O₂ or other reactive oxygen species acts as a second messenger to switch on defense gene expression and activate SAR. However, later studies on the interaction of SA with catalase have provided evidence against the specific binding of SA to plant catalase (16). In addition, while the irreversible catalase inhibitor 3-amino-1,2,4-triazole was found to be a weak inducer of PR-1 expression relative to SA in wild-type tobacco, it was unable to induce PR-1 in nahG transformed plants. This suggests that the action of PR-1 depends on the accumulation of SA but not necessarily on the inhibition of catalase (11, 17).

The present communication is concerned with the interactions of SA with APX in vitro. APX with ascorbic acid (AsA) as a reducing substrate is believed to scavenge excess H₂O₂ formed in plants under both stress and normal conditions (18, 19). The much higher reactivity of APX for hydrogen peroxide compared with catalase and the increase in its activity as a response to several stress conditions indicate a key role for this enzyme in the detoxification of oxygen-activated species (18–20). It was therefore of interest to further investigate the effect of SA on this enzyme. Here we clearly demonstrate that SA is not an effective inhibitor of ascorbate oxidation but is a slow alternative reducing substrate of APX. Possible physiological implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma or Aldrich unless otherwise stated. Reagent grade H₂O₂ as a 30% stock solution was obtained from BDH, and its concentration was determined using an extinction coefficient of 39.4 M⁻¹ cm⁻¹ at 240 nm (21). Freshly frozen tea leaves of Camellia sinensis var. Sinensis clone BBK35 were supplied by Unilever (Colworth House, Bedford, UK) from plants grown in Kenya. Tobacco plants (Nicotiana tabacum cv. Samsun) were grown at 22 °C (John Innes Centre, Norwich, UK) and used for experimentation at 8–10 weeks.

Preparation of Ascorbate Peroxidase—Thylakoid-bound chloroplastic APX isoenzyme was purified to homogeneity from tea leaves using a previously described method (22) with a typical specific activity of 1,500 μmol min⁻¹ mg⁻¹ (assay mixture comprised 1 mM AsA, 0.5 mM H₂O₂, 50 μM FeCl₃, 0.1 M potassium phosphate, pH 7.3, 100 μg chlorophyll). The apoprotein was purified to near homogeneity from chloroplasts by a combination of DEAE-Sepharose and Q-Sepharose chromatography (23).
SA Is Substrate and Not Inhibitor of APX

Effect of SA on APX Activity—The effect of SA on APX activity has been reinvestigated using UV-visible spectrophotometry. The spectrum of SA exhibits two peaks at 290 and 234 nm, while AsA exhibits a single peak at 265 nm. Multiple scans of the reaction of AsA oxidation by APX in the presence of 1 mM SA showed a dramatic time-dependent decrease in absorbance at 265 nm, clearly due to the disappearance of AsA (Fig. 1). When the activities of all of the APX isoenzymes were assayed at 265 nm in a 1-mm cell containing 750 μM AsA, the rates of ascorbate oxidation in the presence and absence of 1 mM SA were not significantly different (1500 ± 50 and 100 ± 5 μmol min⁻¹ mg⁻¹ for chloroplastic and cytosolic ascorbate peroxidases respectively). In addition, ESR measurements showed that similar intensity monodehydroascorbate radical signals were produced by APX both in presence of 10 mM SA and in its absence (Fig. 1). We therefore conclude that SA (10 mM) does not measurably inhibit APX. This conclusion contrasts with that of Durner and Klessig (15) who have reported that SA is an inhibitor of APX. These authors used activity assays monitoring AsA disappearance at 290 nm (Δε = 2.8 μM⁻¹ cm⁻¹) in the presence of SA (1 mM). This assay has been used for APX activity determination for more than a decade since the discovery of the enzyme (24). Nakano and Asada (24) used 290 nm instead of the AsA maximum (265 nm) because of difficulties due to the high absorbance of AsA at the concentrations required to saturate the enzyme. SA absorbs strongly at 290 nm (ε = 3.5 ± 0.05 μM⁻¹ cm⁻¹). The addition of 1 mM SA to the reaction mixture containing 0.75 mM AsA results in absorbance increase from 2.1 to greater than 5.0, while changes in absorbance at 290 nm due to ascorbate oxidation are relatively small (Fig. 1). From Fig. 1 it is clear that the decrease in absorbance at 265 nm rather than at 290 nm should be used for precise measurements of APX activity due to the much lower absorbance of SA at 265 nm. Oxidation of AsA by APX in the presence of SA. Upper panel, time-dependent UV/visible absorption spectra recorded during the oxidation of 750 μM AsA by 0.1 μM tea chloroplastic stromal APX in 50 mM sodium phosphate, pH 7.0, containing 1 mM SA and 2 mM H₂O₂. The broken line is the spectrum of 1 mM SA. The spectra show the decrease in the absorbance peak at 265 nm in the direction indicated by the arrow. Spectra were recorded just before the initiation of the reaction with the enzyme and then at 2, 3, 5, 8, and 12 min. Spectra were recorded using a 1-mm quartz cuvette on a Shimadzu UV-2101PC spectrophotometer, spectral bandwidth of 1 nm, scan speed of 120 nm min⁻¹. Lower panel, X-band ESR signals of monodehydroascorbate radicals generated by APX. ESR signals were recorded by a Bruker ER-200D spectrometer fitted with a room temperature (20–23 °C) flat cell with an attachment allowing rapid loading of samples without demounting the cell. Modulation amplitude, 0.07 mT; modulation frequency, 100 kHz; receiver gain, 10⁶; microwave power, 5.0 milliwatts; microwave frequency, 9.434 Ghz. a, reaction mixture contained 50 mM citrate buffer, pH 4.5, 10 mM AsA, 10 mM H₂O₂, and 0.2 μM tea chloroplastic thylakoid-bound APX. The spectrum was recorded 60–90 s after initiation of the reaction with the enzyme. b, 10 mM SA was added to the reaction mixture. The conditions were exactly the same as in a. No signals were observed for the following samples: ascorbate + peroxide and ascorbate + APX.
absorbance at 257 nm further increases (Fig. 2). The spectrum of the final product is distinguished from SA by its higher absorbance at 257 nm and a shoulder in the region of 340 nm. This spectrum subsequently remained unchanged for more than 1 month.

In an attempt to define the nature of the transient species, ESR experiments using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap were carried out. ESR signals were obtained only in the presence of DMPO, SA, H$_2$O$_2$, and enzyme (data not shown). These signals closely correlated with the formation of an intense brown color. When DMPO was added to the final product of the oxidation, which had a low absorbance at 415 nm, no ESR signals were observed. These results suggest that the initial product of APX-dependent oxidation of the substrate, with its characteristic absorbance peak at 415 nm, may be associated with SA-derived free radicals that undergo subsequent chemistry.

Ascorbate oxidase activity of tea APX was assayed after the initial and final phases of SA oxidation. No enzyme inactivation resulted from SA oxidation. Benzoic acid derivatives such as 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 2,5-dihydroxybenzoic acid were also shown to be reducing substrates and not inhibitors of ascorbate oxidation by tea APX.

DISCUSSION

**SA Is Not an Inhibitor of APX**—The key role of APX in defense of plant cells against hydrogen peroxide is well documented (see Ref. 18 and references therein). With respect to cellular location, ascorbate peroxidases are divided into the following four different types: cytosolic, chloroplast stromal, chloroplast thylakoid-bound, and glyoxysomal (23–26). Most effort to date has gone into the purification and characterization of the first three types of APX. The glyoxysomal form has not been isolated, but its cDNA has been cloned (27). The presence of APX not only in chloroplasts, organelles deficient in catalase, and the cytosol, but also in catalase-rich glyoxysomes suggests that APX functions with catalase as a hydrogen peroxide scavenger. We have studied the effect of SA on cytosolic, chloroplastic thylakoid-bound, and chloroplastic stromal APX isoenzymes purified from tea plants as well as on an APX extract obtained from tobacco plants. Contrary to a recent report (15), we demonstrate conclusively that none of these APX isoenzymes is inhibited by concentrations of SA up to 10 mM. The suggestion that SA-dependent inhibition of APX in planta may result in the elevation of H$_2$O$_2$ levels, which in turn acts as a second messenger in SAR signaling (15), is therefore not tenable. Neuenschwander et al. (17) reported that no increase of H$_2$O$_2$ in tobacco mosaic virus-infected tobacco plants is found during the onset of SAR (17). In addition hydrogen peroxide does not function downstream of SA (11). Taken together with our data, it is clear that APX remains a key antioxidant in plant cells during SAR.

**SA Is a Reducing Substrate of APX**—We have demonstrated for the first time that SA is a reducing substrate of APX. The oxidation reaction is slow apparently due to the presence of an electron-withdrawing carboxyl group adjacent to the hydroxyl group. The transient products of the oxidation appear to be free radicals that undergo subsequent chemistry. This observation has physiological implications since APX-dependent oxidation of SA could be essential for ameliorating the negative effects of this phenolic acid on plant cells. SA is a potential allelochemical, capable of inhibiting seed germination (28), seedling development (29), and mineral uptake by changing the plasma membrane permeability (28). Usually plants tend to detoxify phenolic acids through glycosylation or esterification (30). However, Schulz et al. (28) demonstrated that SA, exogenously applied to roots of Fagopyrum esculentum, was first oxidized to 2,5-dihydroxybenzoic acid and then glucosylated at the 5-OH group. They also found that a protein extract prepared from roots previously treated with SA catalyzed in vitro glucosylation of SA. However, the enzymes responsible for detoxification have not yet been characterized. It is possible that APX and glucosyltransferase act cooperatively. APX-dependent oxidation of SA presumably generates free radicals located at the 1-, 3-, or 5-positions of the benzoic acid ring. Under some circumstances the 5-C$_1$ radical could be hydroxylated and then glucosylated. Apparently, in different plants under certain environmental conditions other products of detoxification of SA may also be formed. Further in vivo studies are necessary to define the mechanism of detoxification of SA by APX.

It has been reported that SA radicals derived from the catalase oxidation-reduction cycle could facilitate lipid peroxidation, which in turn activates PR-1 genes in tobacco cells (14). It is important to note that APX is a much better oxidant of SA than is catalase. However, the induction of PR genes by phenolic acid radicals is not consistent with the observation that 4-hydroxybenzoic acid (also a substrate of APX) does not switch...
on PR protein expression. SA free radicals themselves may play an important role in SAR establishment.

Acknowledgments—M. K. thanks Unilever Research for the supply of freshly frozen tea leaves from Kenya. We are grateful to Prof. J. Draper (University of Leicester, Leicester, UK) and Dr. S. Bornemann (John Innes Center, Norwich, UK) for erudite discussions.

REFERENCES

1. Dempsey, D. A., and Klessig, D. F. (1994) Trends Cell Biol. 4, 334–338
2. Klessig, D. F., and Malamy J. (1994) Plant Mol. Biol. 26, 1439–1458
3. Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990) Science 250, 1002–1004
4. Metraux, J. P., Signer, H., Ryal, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990) Science 250, 1004–1006
5. Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Al-Goy, P., Metraux, J. P., and Ryal, J. A. (1991) Plant Cell 3, 1085–1094
6. Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryal, J. A., and Danogl, J. L. (1994) Cell 77, 565–577
7. Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. (1994) Cell 77, 551–563
8. Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryal, J. (1992) Plant Cell 4, 645–656
9. Gaffney, T., Friedrich, L., Vernocj., B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryal, J. (1994) Plant Cell 6, 959–965
10. Bi, Y. M., Kenton, F., Mur, L., Darby, R., and Draper, J. (1995) Plant J. 8, 235–245
11. Chen, Z., Silva, H., and Klessig, D. F. (1993) Science 262, 1883–1886
12. Conrath, U., Chen, Z., Ricigliano, J. R., and Klessig, D. F. (1995) Plant Biol. 92, 7143–7147
13. Durner, J., and Klessig, D. F. (1996) J. Biol. Chem. 271, 28492–28501
14. Durner, J., and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11312–11316
15. Ruffer, M., Steipe, B., and Zenk, M. H. (1995) FEBS Lett. 377, 175–180
16. Neuchzwander, U., Vernocj., B., Friedrich, L., Uknes, S., Kessmann, H., and Ryal, J. (1995) Plant J. 8, 227–233
17. Asada, K. (1992) Physiol. Plant. 85, 255–241
18. Gara, L. D., Pinto, M. C., Piacella, C., Cappetti, V., and Arrigoni, O. (1996) in Plant Peroxidases: Biochemistry and Physiology (Obinger, C., Burner, U., Ebermann, R., Penel, C., and Greppin, H., eds.) pp. 157–162, University of Agriculture, Vienna, Austria and University of Geneva, Geneva, Switzerland
19. Mittler, R., and Zilinskas, A. (1994) Plant J. 5, 397–405
20. Nelson, D. P., and Kiesow, L. A. (1972) Anal. Biochem. 49, 474–478
21. Kvaratskhelia, M., Winkel C., and Thorneley R. N. F. (1997) Plant Physiol. 114, 1237–1245
22. Chen, G. X., and Asada, K. (1989) Plant Cell Physiol. 30, 987–998
23. Nakano, Y., and Asada K. (1981) Plant Physiol. 67, 867–880
24. Miyake, C., Cao, W. H., and Asada, K. (1993) Plant Cell Physiol. 34, 881–889
25. Schulz, M., Schnabl, H., Manthe, B., Schweihofen, B., and Casser, I. (1993) Phytochemistry 33, 291–294
26. Einhellig, F. A. (1986) in The Science of Allelopathy (Putnam, A. R., and Tang, C. S., eds.) p. 317, Wiley, New York
27. Balke, N. E., Davis, M. P., and Lee, C. C. (1987) in Allelochemicals: Role in Agriculture and Forestry, p. 214, ACS Symposium Series 330, American Chemical Society, Washington, D. C.