Salicylic acid (SA) plays a key role in the establishment of resistance to microbial pathogens in many plants. The discovery that SA inhibits catalase from tobacco led us to suggest that H$_2$O$_2$ acts as second messenger to activate plant defenses. Detailed analyses of SA's interaction with tobacco and mammalian catalases indicate that SA acts as an electron donor for the peroxidative cycle of catalase. When H$_2$O$_2$ fluxes were relatively low (1 μM/min or less), SA inhibited catalase, consistent with its suggested signaling function via H$_2$O$_2$. However, significant inhibition was only observed at 100 μM SA or more, a level reached in infected, but not in uninfected, leaves. This inhibition was probably due to siphoning catalase into the slow peroxidative reaction. Surprisingly, SA was also able to protect catalase from inactivation by damaging levels of H$_2$O$_2$ (lower millimolar range), which is generally assumed to reflect accumulation of inactive ferro-oxo intermediates. SA did so by supporting or substituting for the protective function of catalase-bound NADPH. These results add new features to SA's interaction with heme enzymes and its in vivo redox properties. Thus, SA, in addition to its antioxidant role in containing oxidative processes associated with plant defense responses.

Vertebrate animals possess a novel and highly specific immune system that acts as a defense against disease. Plants react to pathogen attack by activating elaborate defense mechanisms, which are much more poorly characterized than the vertebrate immune system. These defense mechanisms are activated not only at the sites of infection, which are manifested, in part, as necrotic lesions (hypersensitive response; HR), but also in neighboring and even distal uninfected parts of the plant, leading to systemic acquired resistance (SAR). Both HR and SAR are associated with induction of a large number of defense-related genes. The products of these genes may play important roles in the restriction of pathogen growth and spread either indirectly, by participating in strengthening host cellular structures, or directly, by providing antimicrobial activities (for review see Ryals et al. (1994) and Dempsey and Klessig (1995)). During the HR, generation of reactive oxygen species (oxidative burst) precedes formation of necrotic lesions, which result from host cell death (Doke and Ohashi (1988) and Levine et al. (1994); for review see Mehdy (1994)). Additionally, defense responses in surrounding cells become activated, which include synthesis of phytoalexins, pathogenesis-related (PR) proteins, and cell wall polymers such as lignin (Dempsey and Klessig, 1995). Establishment of SAR results in enhanced and long lasting resistance to secondary challenge by the same or even an unrelated pathogen and is associated with activation of PR genes (Ryals et al., 1994; Dempsey and Klessig, 1995). The detailed sequence of molecular events required for the initiation and regulation of HR and SAR is unknown, but progress has been made in identifying several components of the signal transduction pathways leading to disease resistance, among them salicylic acid (SA; for review see Staskawicz et al. (1995) and Dangl (1995)).

SA is present in many plants. While the healing benefits of plants containing high levels of SA have been known since antiquity, the first insights regarding SA's role in plants have emerged only during the past decade. A mounting body of evidence has accumulated that indicates that SA plays an important role in plant defense responses (see review see Ryals et al. (1994); Dempsey and Klessig, 1995). White (1979) was the first to demonstrate that application of exogenous SA or acetylsalicylic acid (aspirin) to tobacco induces PR gene expression and partial resistance to pathogens such as tobacco mosaic virus. Endogenous levels of SA increase dramatically after tobacco mosaic virus inoculation of resistant, but not susceptible, tobacco cultivars and parallel the induction of PR genes (Malamy et al., 1990). In addition, SA induces the same set of nine genes that are activated systemically by tobacco mosaic virus infection (Ward et al., 1991). In cucumber, SA levels rise in the phloem of tobacco necrosis virus-, or Colletotrichum lagenarium-, infected leaves before development of SAR in distal tissues (Métraux et al., 1990). Arabidopsis mutants that develop spontaneous lesions and express genes associated with HR and SAR also have elevated levels of SA (Dietrich et al., 1994; Greenberg et al., 1994). Finally, tobacco mosaic virus-infected transgenic tobacco plants, which express the nahG gene that encodes the SA-metabolizing enzyme salicylate hydroxylase from Pseudomonas putida, accumulate little or no SA, fail to establish SAR, and develop viral lesions that are larger than those produced on wild type plants (Gaffney et al., 1993).

To investigate how SA might function in plant defense responses, our laboratory has focused on the identification of cellular factors with which SA directly interacts. We have suggested that one mechanism of SA action is to inhibit catalase, thereby elevating endogenous levels of H$_2$O$_2$, which result either from the oxidative burst associated with the HR or from metabolic processes such as photosynthesis, respiration, and oxidative phosphorylation (Chen et al., 1993b, 1995). According to this working hypothesis, the elevated H$_2$O$_2$ or other reactive oxygen species derived from it would activate plant defense-related genes such as the PR-1 genes. This mode of activation of plant defenses has been compared with the induc-
tion of genes associated with mammalian immune, inflammatory, and acute phase responses that are mediated through \( \text{H}_{2}\text{O}_{2} \) activation of the transcription factor NF-κB (Schmidt et al., 1995). In support of this model, we have found that (i) 2,6-dichloroisonicotinic acid (INA; a synthetic inducer of PR genes and enhanced resistance) and its biologically active, but not inactive, analogues also inhibit tobacco catalase in vivo (Conrath et al., 1995); (ii) INA as well as SA and its biologically active analogues inhibit the other major \( \text{H}_{2}\text{O}_{2} \)-scavenging enzyme, ascorbate peroxidase, but not guaiacol peroxidases (Durner and Kessler, 1995), and (iii) antioxidants block the action of SA and INA (Conrath et al., 1995).

However, currently there is considerable controversy about the involvement of catalase inhibition by SA and the subsequent increase of \( \text{H}_{2}\text{O}_{2} \) in plant defense responses. Several recent reports have indicated that \( \text{H}_{2}\text{O}_{2} \) is unlikely to be involved in PR gene induction or SAR. Inhibition of catalase in leaf extracts requires concentrations of SA far above those observed in uninfected tissues (Bi et al., 1995; Chen et al., 1993b). In addition, while \( \text{H}_{2}\text{O}_{2} \) and \( \text{H}_{2}\text{O}_{2} \)-inducing chemicals activate PR-1 genes in wild type tobacco, there is little or no gene induction in NahG plants. Therefore, it has been concluded that \( \text{H}_{2}\text{O}_{2} \) induction of SAR genes requires SA rather than the reverse (Neuenschwander et al., 1995). Indeed, it has been shown that very high levels of \( \text{H}_{2}\text{O}_{2} \) stimulate SA biosynthesis (León et al., 1995; Neuenschwander et al., 1995; Sommermanner et al., 1995). Taken together, these results suggest that \( \text{H}_{2}\text{O}_{2} \) does not function downstream of SA (i.e. by inhibition of catalase) in the regulation of PR genes.

However, catalase is still the subject of many mechanistic investigations. There is increasing evidence that catalase is a major factor in a variety of pathological states such as cancer, diabetes, aging, and oxidative stress (see DeLuca et al. (1993)). Inactivation and reactivation of catalase in vivo and in vitro are far from being fully understood. Numerous recent publications suggest new approaches regarding in vitro assays and inhibition studies on catalase (Feuers et al., 1993; DeLuca et al., 1995; Ou and Wolff, 1994, 1996; Escobar et al., 1996; Hook and Hardinge, 1996). In the present report, we provide new insights into SA’s effects on catalase. SA acts as an electron donor for the peroxidative cycle of both plant and animal catalases. As such, it can protect as well as inhibit catalase activity, depending on the concentration of \( \text{H}_{2}\text{O}_{2} \). It is hypothesized that, in healthy tissue of infected leaves where \( \text{H}_{2}\text{O}_{2} \) levels are low, SA inhibits catalase, which could lead to activation of defense-related genes. In contrast, in infected cells and in tissue immediately adjacent to necrotizing cells, where high levels of \( \text{H}_{2}\text{O}_{2} \) and other reactive oxygen species are produced, SA protects catalase from inactivation. This property of SA might serve to contain the oxidative damage associated with spread of the lesion and resembles closely some antioxidantive properties of SA in activated HeLa cells or inflamed mammalian tissues, which are unrelated to inhibition of prostaglandin H synthase.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Tobacco plants (Nicoitana tabacum cv. Xanthi nc) were grown at 22 °C in growth chambers programmed for a 14-h light and 10-h dark cycle. 6–8-week-old plants were used for experimentation.

**Chemicals and Enzymes**—SA, SA analogues, simple phenolic compounds, phlorizin (phloretin-2′,3′-O-b-glucoside), and bunetamide (3-n-butyramino-4-phenoxy-5-sulfonyl-benzamide) were purchased from Sigma or Aldrich. INA was kindly provided by Helmut Kessmann of CIBA-Geigy Ltd. (Basel, Switzerland). Catalase (\( \text{H}_{2}\text{O}_{2} \)/H\( _{2}\text{O} \) oxidoreductase; EC 1.11.1.6) from **Aspergillus niger** was filtered through four layers of cheesecloth and centrifuged at 10,000 \( g \), 10 min.

**Enzyme Assays**—Catalase activity was measured in 50 mM potassium phosphate, pH 6.6, with a commercial oxygen electrode (model 5739; Yellow Springs Instruments, Yellow Springs, OH). After washing with 20 mM potassium phosphate, pH 6.6, the sample was applied to a hydroxyapatite column (1 m, 35 cm) that was equilibrated with 20 mM potassium phosphate, pH 6.6, at 24°C. Prior to application, the sample was centrifuged (10,000 \( g \), 10 min). After buffer exchange (NAP-10, Pharmacia Biotech Inc., equilibrated with 25 mM potassium phosphate, pH 6.6), the protein concentration was adjusted to 0.5 mg/ml.

**Spectrophotometry**—To analyze the redox states of catalase, spectra in the near UV region (Soret region, 340–450 nm) were scanned with a Beckman DU-7 spectrophotometer, using 1-mL semimicro black side-wall quartz cuvettes. The binding of NADPH by tobacco and mammalian catalase was assayed by fluorescence spectroscopy using a LS-3B/R
100A system (Perkin-Elmer) and 0.5-ml fluorescence cells. After excitation at 360 nm, emission spectra from 360–560 nm were recorded. Immediately before the measurements, commercially available enzymes were treated as described under “Enzyme Assays.”

Electrophoretic Techniques—SDS-PAGE was performed with 10% (2.7% cross-linker) gels. Gels were stained with Coomassie Blue R-250 or with silver nitrate using the Bio-Rad silver stain kit. For immunoblotting, proteins were transferred to a nitrocellulose membrane, and catalase was detected with a mixture of monoclonal antibodies (MAb3B6 and MAb1F5) made against tobacco catalase and with the ECL detection kit from DuPont (Chen et al., 1993b).

Horizontal isoelectric focusing was carried out on Ampholine gels, pH 5.5–8.5 (Pharmacia), at 50 mA for 1 h (270–1100 V), followed by 1650 V for 1.5 h. The samples were prepared by grinding tissue under liquid nitrogen (0.2 g/ml of extraction buffer as described for the large scale extraction of catalase). After centrifugation for 10 min, the homogenate was desalted (NAP-5) against 10 mM potassium phosphate, pH 6.6. After focusing, gels were negatively stained for catalase activity using horseradish peroxidase and 3,3′-diaminobenzidine as described by Mullen and Gifford (1993).

RESULTS

Separation of Catalase Isoforms—The discovery that the SA-binding protein from tobacco was a catalase whose activity was inhibited by SA (Chen et al., 1993b) prompted the question as to whether only a subset of the isoforms of this enzyme are sensitive to SA. In contrast to mammals, in plants catalase is encoded by a small gene family (Ni and Trelease, 1991; Scandalios, 1994; Willekens et al., 1995). The active enzyme is a tetramer made up of four identical or similar subunits encoded by the same or different family members, respectively. Therefore, plants contain multiple isoforms of this enzyme. In tobacco, Zelitch et al. (1991) identified at least 6 different isoforms, while Siminis et al. (1994) detected 6–12 isoforms. We have extended this isoform analysis using purified tobacco leaf catalase. When purified tobacco catalase was subjected to analytical chromatofocusing on a Mono P column, multiple peaks were obtained (Fig. 1A). It should be noted that the use of purified catalase allowed for direct detection of the isoforms (absorbance at 280 nm), in contrast to an indirect approach based on an activity profile. At least 10 catalase species eluted between pH 7.6 and 6.0. SDS-PAGE analysis indicated that the most basic and most acidic isoforms consisted exclusively of larger (57-kDa) and smaller (55-kDa) subunits, respectively, whereas some intermediate isoforms in fractions 15–17 appeared to be heterotetramers (visible in the inset of Fig. 1B). Interestingly, the specific activity of homotetramers consisting only of 57-kDa subunits was much higher (124 units mg⁻¹ for the pooled fractions 8–11, hereafter referred to as pool 1) than that of isoforms containing only the 55-kDa subunits (18.4 units mg⁻¹ for the pooled fractions 19–21, hereafter referred to as pool 2). As a consequence, the elution profile shown in Fig. 1A does not reflect the distribution of catalase activity among the various isoforms (Fig. 1C).

The relative catalase activity of selected fractions, as well as their sensitivity to SA is shown in Fig. 1C. Isoforms consisting only of large subunits accounted for 75–80% of the overall catalase activity. Catalase activity throughout the tested fractions was inhibited 36–51% by 1 mM SA. Thus, there appears to be little difference in SA sensitivity between isoforms, regardless of their subunit composition.

In addition to degradation of H₂O₂ to H₂O and O₂, catalase has a peroxidative activity (see Fig. 3); the ratios of these two activities can differ substantially among isoforms (Havir and McHale, 1987; Zamotoy et al., 1995). For tobacco leaf catalase, the ratios of the peroxidative activity to the catalatic activity were 0.42 × 10⁻⁴ for pool 1 and 4.5 × 10⁻⁴ for pool 2. Note that the peroxidative-like activity was measured with ethanol rather than with true peroxidase substrates such as guaiacol or pyrogallol, which are poor electron donors for catalase.
and did not contain the acidic isozymes (Fig. 2B). In ASCAT1 plants, the larger (57-kDa) subunit was absent. As a consequence, the majority of basic and neutral isoforms was missing. In agreement with the isoform pattern shown in Fig. 1, the larger subunit encoded by cat1 assembles into at least five different isoforms. The activity profile of the chromatofocused, purified catalase (Fig. 1C) mimics the catalase activity staining pattern of isoelectric focused crude extracts (Fig. 2B), with the majority of the activity represented by isoforms with neutral or basic isoelectric points and consisting of 57-kDa subunits. Therefore, the majority of the isoforms and their relative abundance appear to have been retained during purification.

Mode of Action of SA on Catalase—The first step in the catalase cycle (Fig. 3) involves a two-electron (e\textsuperscript{\text{-}}) equivalent reduction of H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O and the corresponding oxidation of ferric enzyme (ferricatalase) to compound I, a spectriscopically distinct and enzymatically active form of catalase (for review see Deisseroth and Dounce (1970); Schonbaum and Chance (1976)). Compound I is converted back to ferricatalase by a 2e\textsuperscript{\text{-}} equivalent reduction and the corresponding oxidation of a second molecule of H\textsubscript{2}O\textsubscript{2} to O\textsubscript{2} (step 2), thus completing the catalatic (e\textsuperscript{\text{-}}) cycle. In the peroxidative (\(\beta\)) cycle of catalase, compound I is converted to compound II by a 1e\textsuperscript{\text{-}} equivalent reduction (step 3). Compound II is inactive with respect to the catalatic cycle and has a different absorption spectrum than ferricatalase or compound I (optimum at 432 nm versus 405–409 nm). Through a second 1e\textsuperscript{\text{-}} equivalent reduction, compound II can be converted back to ferricatalase (step 4). AH represents an electron donor (e.g., a phenolic compound), while \(\Delta^\text{z}\) denotes the resulting radical formed after donation of an electron. Compound III is an inactive form of catalase produced at high H\textsubscript{2}O\textsubscript{2} levels, which is not readily reverted back to compound II.

In order to more closely mimic the in vivo situation where H\textsubscript{2}O\textsubscript{2} is almost continuously being produced, low to moderate levels of H\textsubscript{2}O\textsubscript{2} were generated at a prescribed rate during the catalase reaction using various concentrations of glucose and glucose oxidase. The effects of continuous H\textsubscript{2}O\textsubscript{2} fluxes on catalase (inactivation) have been studied in detail by several groups (Kirkman et al., 1987; Hillar et al., 1994; Ou and Wolff, 1996). This H\textsubscript{2}O\textsubscript{2}-generating system, however, could not be used to attain high levels of H\textsubscript{2}O\textsubscript{2}, since all commercial glucose oxidase preparations contain low levels of other contaminating enzymes that affect O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} metabolism. Therefore, reaction mixes were adjusted to millimolar levels of H\textsubscript{2}O\textsubscript{2} with 30% H\textsubscript{2}O\textsubscript{2} stocks at the start of the reaction as described by Deisseroth and Dounce (1970). Although an initial H\textsubscript{2}O\textsubscript{2} concentration of 10 mM may seem high, it was used routinely by Chance and others (reviewed by Schonbaum and Chance (1976)) to study inactivation of catalase in vitro. Under these conditions, high H\textsubscript{2}O\textsubscript{2} levels are maintained for only short periods after the addition of catalase; however, this method is commonly used to assay the susceptibility of catalase to H\textsubscript{2}O\textsubscript{2} (Feuers et al., 1993; DeLuca et al., 1995).

It is well established that catalase is inactivated by its own substrate, H\textsubscript{2}O\textsubscript{2} (Kirkman et al., 1987; DeLuca et al., 1995). This is, in part, due to H\textsubscript{2}O\textsubscript{2}-mediated accumulation of compound II (at low H\textsubscript{2}O\textsubscript{2} concentrations) and conversion of compound II to compound III (at high H\textsubscript{2}O\textsubscript{2} concentrations), an inactive form of catalase that cannot be easily converted back to active enzyme (Fig. 3; see Schonbaum and Chance (1976)). The rate and amount of inactivation rises with increasing levels of H\textsubscript{2}O\textsubscript{2}, as shown for the control in Fig. 4 and described previously (Kirkman et al., 1987). At a relatively low rate of
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H$_2$O$_2$ production (0.1 nmol ml$^{-1}$ min$^{-1}$), the addition of SA (0.2 mM) inhibited catalase about 50% compared with the control, which exhibited only very limited activity loss during the 200-min incubation. When the rate of H$_2$O$_2$ generation was increased 10-fold, SA accelerated the inactivation of catalase by H$_2$O$_2$. However, eventually (at ~200 min) the catalase activity dropped to approximately 50% of the initial level, regardless of the presence of SA. At high levels of H$_2$O$_2$ (i.e. an initial concentration of 10 mM), SA again initially accelerated the rate of catalase inactivation, but as the reaction time increased, SA protected the enzyme against further inactivation. Because of the different kinetics of catalase inactivation by H$_2$O$_2$ and SA, dramatically different results were obtained with respect to the effects of SA on catalase activity depending on the concentration of H$_2$O$_2$ present. This is illustrated in Fig. 5, A and B, in which the effect of SA (0.2 mM) on purified tobacco catalase was measured after a 3-h incubation at different levels of H$_2$O$_2$. Qualitatively similar results were obtained after a 1-h incubation (data not shown). Note that the values are given in percentage of catalase activity of a control without SA. Means ± S.E. are shown, with n = 3.

To help elucidate how SA can both inhibit and activate catalase, we examined the effects of SA on formation of the various redox states or reaction intermediates of catalase (Fig. 3) that can be distinguished spectroscopically by their absorption spectra in the Soret (near UV) region. The absorption spectrum in the Soret region of the purified enzyme is shown in Fig. 6A (curve 1). The broad peak centered around 405 nm is consistent with the enzyme existing primarily in the ferricatalase (Fe(III)) state. After incubation of the enzyme with H$_2$O$_2$ (generated at 1 nmol ml$^{-1}$ min$^{-1}$) for 1 h, the curve flattened slightly (curve 2), consistent with the conversion of some of the ferricatalase to compound I (Fe(V)), which has a lower extinction coefficient than ferricatalase. In contrast, when the enzyme was incubated with H$_2$O$_2$ plus 0.2 mM SA for 1 h, there was increased absorbance at 420–440 nm, which is characteristic of compound II, and decreased absorbance at 405 nm, consistent with a reduction in the amount of ferricatalase and consistent with the conversion of compound I to compound II (Fe(V)). The spectral shifts shown in Fig. 6 resemble those published by others (e.g. Jouve et al. (1986) and Hillar et al. (1994)) but are somewhat less pronounced than those obtained for pure compound II (Schonbaum and Chance, 1976; Deisseroth and Dounce, 1970). However, this spectral change induced by SA suggests that it is serving as an electron donor for conversion of compound I to compound II (Fig. 3, step 3).

The ability of SA to serve as an electron donor for conversion of compound II back to ferricatalase (Fig. 3, step 4), thus completing the peroxidative cycle, was then analyzed. To address this question, we chose conditions in which some of the enzyme was trapped in the compound II state and then determined whether the addition of SA would convert the trapped compound II to ferricatalase. A similar approach has been carried out in order to show the effect of NADPH on bovine catalase.

Fig. 5. Effect of SA on catalase activity in the presence of varying concentrations of H$_2$O$_2$. Catalase (4.8 μg ml$^{-1}$ or 0.08 μM, pool I from Fig. 1) was incubated with or without SA (0.2 mM) for 3 h in the presence of H$_2$O$_2$. In A, H$_2$O$_2$ production given in nmol ml$^{-1}$ min$^{-1}$ was achieved with the glucose/glucose oxidase system. In B, H$_2$O$_2$ concentrations (in μmol ml$^{-1}$) were achieved by the addition of H$_2$O$_2$ at 0.5, 5, and 20 min. After 3 h, catalase activity was measured as described under “Enzyme Assays.” Values are expressed as percentage of catalase activity of the corresponding control without SA. Means ± S.E. are shown, with n = 3.
Fig. 6. SA acts as an electron donor for the peroxidative cycle of catalase. A, formation of the enzyme intermediate compound II (Fe(IV)) of catalase by SA. Trace or curve 1 is for ferric (Fe(III)) catalase (2 μM). Trace 2 shows formation of compound I in the presence of 0.4 mM H₂O₂ generated by glucose oxidase at a rate of 1 nmol ml⁻¹ min⁻¹. The spectrum was obtained after 1 h. For trace 3, SA (0.2 mM) was added to catalase along with glucose oxidase. It shows the formation of compound II. Trace 1 is the top curve, while trace 3 is the bottom curve at 405 nm. B, formation of the ferric form (Fe(III)) of catalase by SA. Catalase (2 μM) was partially inactivated by the repeated addition of H₂O₂ (the addition of H₂O₂ to 250 μM three times over 20 min, total incubation time 30 min); this resulted in trace 1. Traces 2–4 correspond to scans taken 5, 10, and 15 min, respectively, after the addition of 0.2 mM SA. Trace 1 is the bottom curve, while trace 4 is the top curve at 405 nm. See “Results” for discussion.

(Souave et al., 1986; see below). During the catalytic cycle (Fig. 3, steps 1 and 2), a small amount of compound I can be spontaneously converted into compound II (step 3), even within a short period and even in the absence of an electron donor (Schonbaum and Chance, 1976; Kirkman et al., 1987; Hillar et al., 1994; Deluca et al., 1995). Since this spontaneous generation of compound II is low compared with that seen when an electron donor like SA is present, it is difficult to follow compound II formation as an increase in absorbance at 420–440 nm. However, its formation can be surmised from a modest reduction in absorbance at 405 nm and a small shift to longer wavelengths. To facilitate spontaneous formation of compound II, purified tobacco enzyme was allowed to react with modest levels of H₂O₂ (250 μM) three times. Thus, H₂O₂ was extensively provided as described recently (DeLuca et al., 1995) rather than generated by glucose oxidase as reported by Kirkman et al. (1987). The reduction in absorbance at 405 nm of the reacted catalase mixture is evident when curve 1 in Fig. 6B is compared to curve 1 in Fig. 6A, which represents the absorbance of purified catalase before reacting with H₂O₂. It should be noted, however, that in the presence of small amounts of H₂O₂, catalase will exist as a mixture of all intermediates. The addition of 0.2 mM SA to the reacted catalase mixture without the addition of more H₂O₂ led to increased absorbance at 405 nm (Fig. 6B, curves 2–4). This increase is consistent with reformation of ferricatalase (Jouve et al., 1986). Thus, SA must act as an electron donor for compound II, as well as for compound I.

Interestingly, two of the three commercial preparations of mammalian catalase (one of two bovine liver preparations and one from human erythrocytes), showed similar increases in absorbance at 405 nm with the addition of SA, even without pretreatment with H₂O₂ (data not shown). This is reminiscent of the observation of others that many catalase preparations consist of a mixture of intermediates (Deisseroth and Dounce, 1970; Zamocky et al., 1995). We conclude from these results that SA can serve as electron donor for the peroxidative or β activity of plant and animal catalases.

Many catalase inhibitors, representing different classes of chemicals, have been reported, and the modes of action of several of these, such as 3-amino triazole, resorcinol, ascorbate, and dithiothreitol have been described (for review see Schonbaum and Chance (1976)). The efficiency of inhibition of tobacco catalase by SA and related chemicals is compared with that of various traditional catalase inhibitors in Table I. This data also allows for a pharmacological comparison of tobacco catalase with published results on catalase from other species. In general, chemicals that are biologically active for induction of PR-1 gene expression and enhanced disease resistance in plants, which include SA, aspirin, 2,6-dihydroxybenzoic acid, 4-chloro-SA, 3-chloro-SA, 3,5-chloro-SA, and benzoic acid (Chen et al., 1993a, 1993b; Gaffney et al., 1993; Conrath et al., 1995). A 0.08 μM catalase (pool 1 from Fig. 1) was incubated for 1 h in presence of 0.4 mM inhibitor, except where indicated differently. H₂O₂ production by the glucose/glucose oxidase system was adjusted to 1 nmol ml⁻¹ min⁻¹. Catalase activity was measured as described. Means ± S.E. are shown, with n = 3. Similar results were obtained for SA, catechol, and INA with pool 2, which consisted of isoforms made up of subunits encoded by the cat2 gene.

### TABLE I

| Compound*  | Percentage of inhibitionb | Compound  | Percentage of inhibition |
|------------|---------------------------|-----------|-------------------------|
| SA         | 50 ± 9                    | INA       | 0                       |
| Aspirin    | 39 ± 3                    | Resorcinol| 94 ± 5                  |
| 2,6-DHBAa  | 72 ± 10                   | Pyrogallol| 93 ± 5                  |
| 4Cl-SA     | 35 ± 8                    | Hydroquinone| 88 ± 9                 |
| 3Cl-SA     | 53 ± 3                    | 3-Aminotriazole| 35 ± 20f                |
| 3,5Cl-SA   | 40 ± 10                   | 2-Mercaptotoluol| 31 ± 4                  |
| 3-HBAa     | 0                         | Dithiothreitol| 24 ± 9                  |
| 4-HBA      | 3 ± 4                     | Ascorbate | 40 ± 23d                |
| Benzoic acid| 41 ± 2                   | Phlorizin | 26 ± 5                  |
| Catechol   | 80 ± 4                    | Bumetanide| 84 ± 9f                 |

* SA, aspirin, 2,6-DHBA, 4Cl-SA, 3Cl-SA, 3,5-Cl-SA, benzoic acid, and INA are biologically active for induction of PR gene expression and disease resistance, while 3-HBA, 4-HBA, and catechol are not (Chen et al., 1993a, 1993b; Gaffney et al., 1993; Conrath et al., 1995). ** A 0.08 μM catalase (pool 1 from Fig. 1) was incubated for 1 h in presence of 0.4 mM inhibitor, except where indicated differently. H₂O₂ production by the glucose/glucose oxidase system was adjusted to 1 nmol ml⁻¹ min⁻¹. Catalase activity was measured as described. Means ± S.E. are shown, with n = 3. Similar results were obtained for SA, catechol, and INA with pool 2, which consisted of isoforms made up of subunits encoded by the cat2 gene.

**DHA, dihydroxybenzoic acid.**  
**2 mM.**  
**1 mM.**  
**HBA, hydroxybenzoic acid.**  
**0.1 mM.**

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* M. D. Anderson, Z. Chen, and D. F. Klessig, unpublished results.
inhibition of catalase, an alternative explanation is that INA needs to be metabolized to an active form that both inhibits catalase and induces defense responses. This view is supported by the observation that INA is a very effective inhibitor of tobacco catalase in vivo, while in crude tobacco extracts its inhibition is less pronounced (Conrath et al., 1995), and is consistent with the finding that labeled INA is partially converted to another compound after injection into plants (Métraux et al., 1991).

In addition to traditional catalase inhibitors, SA, and related chemicals, two drugs, bumetanide and phlorizin, were analyzed for their effect on catalase (Table I). Both compounds have recently been reported to interact with mammalian catalase. The diuretic drug, bumetanide, is an inhibitor of the mammalian Na+/K+/Cl− cotransporter and has recently been shown to bind to membrane-associated catalase from liver (Ottallah-Kolac, 1995). Bumetanide very effectively inhibited tobacco catalase (activity was inhibited by more than 50% with as little as 50 μM). Phlorizin is an inhibitor of another cotransporter, the Na+/glucose cotransporter from kidneys, and it binds to the NADPH-binding site of mammalian catalase (Kitlar et al., 1994). This drug also inhibited tobacco catalase, but it was less effective than bumetanide.

Tobacco Catalase Has NADPH-binding Sites—The discovery that bovine catalase contains tightly bound NADPH (Kirkman et al., 1987) together with the identification of the NADPH-binding sites within the three-dimensional structure of this enzyme (Fita and Rossman, 1985) prompted a search for other catalases with NADPH-binding sites. While several other animal catalases have been shown to bind NADPH (Kitlar et al., 1994; Hillar et al., 1994), to date NADPH-binding catalases have not been detected in plants. However, the inhibition of tobacco catalase by phlorizin and the affinity of the enzyme for the NADPH analogue Cibacron blue (Chen et al., 1993a) led us to investigate whether purified tobacco catalase could bind NADPH. We employed the spectrometric method of Hillar et al. (1994), which depends on the fluorescence of bound NADPH after excitation at 340 nm. Commercial preparations of bovine catalase, which contained bound NADPH (Kirkman et al., 1987), were used as a positive control. After excitation, bovine catalase exhibited a peak of fluorescence centered at 430 nm (Fig. 7, curve 1b). Pretreatment of this catalase with NADPH to saturate the binding sites increased the fluorescence, as expected (curve 1a). Purified tobacco catalase gave a similar but lower peak of fluorescence (curve 2b), which was again elevated by pretreatment with NADPH (curve 2a). In contrast, catalase from A. niger, which does not have NADPH-binding sites (Hillar et al., 1994), did not show any NADPH-related fluorescence, with or without pretreatment (curves 3b and 3a, respectively). These results suggest that tobacco catalase contains NADPH-binding sites.

Activity of Tobacco and Bovine Catalases Is Protected by NADPH and Modulated by SA—Kirkman et al. (1987) have shown that NADPH protects mammalian catalases from H2O2 inactivation, which can occur even at low concentrations of H2O2. Since tobacco catalase also contains NADPH-binding sites, it was of interest to know if this cofactor could also protect the plant catalase from inactivation by H2O2. In Fig. 8A, incubation of tobacco or bovine catalase in the presence of a relatively low rate of H2O2 production (1 nmol ml−1 min−1) for 1 h resulted in a slight loss of activity, whereas in the presence of 4 μM NADPH (supplied initially as NADPH and maintained by a regenerating system) catalase activity was enhanced. Since catalase is purified in the absence of exogenous NADPH, the enhancement of initial activity by NADPH may be due to saturation of the NADPH-binding sites. With 0.2 mM SA, catalase (both bovine and tobacco) was inhibited 35–45%, regardless of the presence of NADPH, indicating that SA and NADPH are affecting catalase differently. At a high concentration of H2O2 (10 μmol ml−1, initial concentration), catalase activity of the controls (without SA and NADPH) decreased dramatically (Fig. 8B; also see Fig. 4C), again likely due to formation of compounds II and III. Both SA and NADPH protected catalase to some extent, and when applied together, the protective effect was additive. Thus, at low H2O2 levels, SA inhibits both enzymes, while at high levels of H2O2, it protects them from almost complete inactivation. In contrast, NADPH protects both enzymes regardless of the H2O2 concentration. These data are consistent with the proposed role of SA as an electron donor for the β activity of catalase and with the protective effect afforded by the binding of NADPH to catalase (Kirkman et al., 1987).

**DISCUSSION**

Catalase was one of the first enzymes to be purified and crystallized (see Schonbaum and Chance, 1976). However, despite extensive biophysical, biochemical, and genetic analyses, there is an ongoing discussion as to whether the only, or even major, role of this very abundant protein is to convert H2O2 to H2O and O2 (its catalatic or α activity). This may, in part, reflect the complexity of catalase’s redox chemistry. In recent years, catalase has gained renewed attention. There is increasing interest in the involvement of oxidative stress in environmental pollution, aging, diabetes, cancer, and other human diseases and in catalase’s role as one of the main antioxidative enzymes. In particular, this has led to renewed interest in the mechanism of catalase inhibition and inactivation (Feuers et al., 1993), Hillar et al. (1994), DeLuca et al. (1995), Ou and Wolff (1994, 1996), Escobar et al. (1996), and references therein).

**SA Inhibits All Catalase Isoforms from Tobacco Leaves**—In plants, catalase is encoded by a small gene family consisting of several classes (Ni and Trelease, 1991; Scandalias, 1994; Willekens et al., 1995). This leads to multiple isoforms of the enzyme, since individual subunits encoded by different family members can homo- or heterotetramerize (Ni and Trelease,
However, while we could not detect a tobacco catalase in rice (Sánchez-Casas and Klessig, 1994). Furthermore, SA did not inhibit purified maize catalase (Guan and Scandalios, 1995).

**Mechanism of SA Action on Tobacco and Mammalian Catalases**—The biphasic kinetics of catalase inhibition by SA (Fig. 4) have been reported for other phenolic compounds such as hydroquinone and pyrogallol and have been interpreted as the transition from the fast catalatic or α activity to the slow peroxidative or β activity (Goldacre and Galston (1953), Ogura *et al.* (1950); reviewed by Schonbaum and Chance (1976); see Fig. 5). This type of inhibition is clearly different from the time-dependent and mechanism-based processes caused by inhibitors such as aminotriazole (Schonbaum and Chance, 1976). Further evidence that SA acts as a typical phenolic by stimulating the peroxidative activity of tobacco catalase at the expense of its catalatic activity was provided by spectral analysis of catalase and its reaction intermediates (Fig. 6). Together, these analyses indicate that SA acts as an electron donor for the enzyme intermediates compound I and compound II. This is consistent with previous studies, which demonstrated that phenolics can reduce compound I to compound II and compound II to the ferric enzyme (reviewed by Deisseroth and Dounce (1970); Schonbaum and Chance (1976)) and the more recent reports that SA or aspirin can act as an electron donor for myeloperoxidase and horseradish peroxidase (Kettle and Winterbourn, 1991; Durner and Klessig, 1995). In other words, SA inhibits catalase by acting as a one-electron donor that siphons compound I from the extremely fast catalatic cycle (see Fig. 3) into the relatively slow peroxidative cycle (~1000 times slower) (Havir and McHale, 1987; Zamocky *et al.*, 1995) by promoting the formation of compound II. It is noteworthy that Luck (1957) and Itoh *et al.* (1962), as part of their studies on the effects of carboxylic acids on catalase, were the first to demonstrate that SA (at extremely high levels of ~10 mM) inhibited mammalian catalases and to speculate that the inhibition probably resulted from promotion of the peroxidative reaction rather than from chelation of the heme iron of catalase as has been suggested by others (e.g. Rüffer *et al.*, 1995). Very recently, Russell and Sternberg (1996) proposed that catalase contains a novel binding site on its surface based on structural similarities to the calycin superfamily. They suggested that SA inhibits catalase by binding to this site and causing a conformational change (allosteric inhibition). While such a site is not inconsistent with data presented here, our results argue that SA inhibits catalase by acting as an electron donor rather than by inducing a conformational change. SA could bind to this surface site and still act as an electron donor to the deeply buried heme of catalase, since Bonagura *et al.* (1996) have demonstrated that an electron donor at the surface of peroxidases can transfer electrons indirectly to the heme.

The ability of SA to inhibit bovine catalase contrasts with our preliminary analysis, which suggested that mammalian catalases were not inhibited by SA (Chen *et al.*, 1993b). It also illustrates the difficulties that can be encountered when determining the effects of potential inhibitors like SA on this complex enzyme, whose reaction chemistry is still debated. H₂O₂ itself can dramatically alter the effects seen with SA as illustrated in Figs. 4, 5, and 8. This likely is responsible for some of the discrepancy in results recently reported (Sánchez-Casas and Klessig, 1994; Summermatter *et al.*, 1995). The standard assay in which the rate of H₂O₂ utilization is measured only for a few minutes (Aebi, 1984) is adequate for determining relative catalase activity in different tissues. However, it is poorly suited to analyze the effects of potential inhibitors on catalase.

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1 Z. Chen and D. F. Klessig, unpublished results.
activity. Inhibition by phenolics is time-dependent and requires H$_2$O$_2$ (Fig. 4), just as has been previously described (Ogura et al., 1950). Other less readily controlled variables include the presence of phenolics in crude extracts, which can lead to partial inactivation of catalase through formation of compound II (DeLuca et al., 1995) and thus to an underestimation of the inhibitory potential of a phenolic such as SA.

**Does SA Modulate Catalase Activity in Vivo?—**In infected tissues, SA levels can approach 100 $\mu$M (Malamy et al., 1990 and 1992; Enyedi et al., 1992), a concentration sufficient to cause a considerable inhibition of catalase and ascorbate peroxidase (Bi et al., 1995; Conrath et al., 1995; Durner and Klessig, 1995).

Because of catalase’s unique feature that it is inactivated by its own substrate (Fig. 4) (Hillar et al., 1994; DeLuca et al., 1995), even modest effects on the activity of these two major H$_2$O$_2$-scavenging enzymes could feed back to cause further inactivation of catalase by the slow and time-dependent accumulation of H$_2$O$_2$.

The role of catalase and SA in uninfected parts of an infected plant is considerably less clear. While SA also accumulates in these tissues, the level appears to be far below the concentration required to effectively inhibit catalase and ascorbate peroxidase (Malamy et al., 1990; Enyedi et al., 1992).

SA’s role in SAR development is unlikely to involve elevated levels of H$_2$O$_2$, resulting from its inhibition of catalase, as originally proposed, unless SA is highly concentrated in certain subcellular compartments (Chen et al., 1993b). Nonetheless, SA induction of SAR may be mechanistically coupled to its interaction with catalase and peroxidases. SA serves as a one-electron donor for catalase (Fig. 6) and peroxidases (Durner and Klessig, 1995) and in so doing is converted to a free radical. Free radicals of phenolics (e.g. Savenkova, et al. (1994)) can initiate formation of lipid peroxides. Our preliminary studies indicate that SA induces lipid peroxidation, while several naturally occurring lipid peroxides activate PR-1 genes in tobacco cells. A free radical could result in the formation of an effective lipid peroxide signal without readily discernible inhibition of catalase. However, the biological significance of a SA radical generated by catalase remains to be proven.

In addition to its ability to inhibit catalase, SA could also protect plant and mammalian catalases against inactivation by H$_2$O$_2$ in vitro (Figs. 4C, 5, and 8). This is functionally similar to the protective effects of NADPH on mammalian catalases in the presence of small fluxes of H$_2$O$_2$ as described by Kirkman et al. (1987). Indeed, we found that tobacco catalase, like mammalian catalases, contains bound NADPH (Fig. 7). Therefore, it appears that, under some conditions, SA can support or substitute for NADPH’s protective role. Might it serve a similar function in vivo? In animal systems accumulation of compound II (and thus catalase inhibition) has been associated with “abnormal” stress conditions such as found in tumors or during prolonged hypoxia or cell necrosis (Oshino et al., 1973). Furthermore, reactive oxygen species produced by NADPH oxidase, induced by tumor necrosis factor $\alpha$, causes significant decrease in rat hepatic catalase activity (Yasmin et al., 1991). In the case of plants, similar stress conditions may occur during necrotic lesion formation in the HR. A strong oxidative burst (probably produced by a NADPH oxidase) is associated with the HR (Doke and Ohashi, 1988; Orlandi et al., 1992; Levine et al., 1994), which could result in catalase inactivation by $\mathrm{O}_2^*$ and H$_2$O$_2$ (Schenbaum and Chance, 1976; Kono and Fridovich, 1982). Catalase inactivation during the HR would be enhanced by the proposed depletion of NADPH by NADPH oxidases and antioxidative enzymes of the ascorbate/glutathione cycle (Mehdy et al., 1994). One might speculate that under these conditions, SA may protect or reactivate a basal catalase activity. This notion is consistent with the observation that SA appears to act as an antioxidant at sites of inflammation in animals (Halliwell et al., 1988); one property of SA may be to maintain a basal level of catalase activity by acting as an electron donor that converts inactive compound II to the active ferricatalase (Fig. 6). In fact, it has been suggested that SA protects various heme proteins such as leghemoglobin and metmyoglobin from H$_2$O$_2$-induced inactivation by maintaining the peroxidative cycle of these $\mathrm{O}_2^*$-binding proteins (Galaris et al., 1988; Pupplo and Halliwell, 1988). Alternatively, SA may serve as a quencher of radicals associated with the heme group of inactivated catalase or other heme proteins (Galaris et al., 1988; Pupplo and Halliwell, 1988). SA is a direct scavenger of OH$^-$ (in vivo and in vitro), and it is a iron-chelating compound, thereby inhibiting the direct impact of OH$^-$ as well as its generation via the Fenton reaction (Halliwell et al., 1995). However, since desferrioxamine (a strong chelating agent) did not protect catalase from inactivation, we hypothesize that SA maintains a basal catalase activity through its ability to serve as an electron donor.

In sum, whether SA positively or negatively modulates catalase activity will depend on the redox status of the cell. In the healthy tissue surrounding, but not immediately adjacent to, the infection site H$_2$O$_2$ concentrations will be relatively low to moderate, and the elevated SA levels probably inhibit catalase by promoting the slow peroxidative cycle (note that in normal healthy leaf tissue H$_2$O$_2$ has been estimated at $\sim 100$ nM; Scandalios (1994)). The resultant increase in H$_2$O$_2$ could serve as a second messenger to facilitate activation of plant defense genes.

In contrast, in the infected cells and immediately adjacent tissue, high levels of reactive oxygen species resulting either from the oxidative burst associated with the HR (Doke and Ohashi, 1988) or from long lasting oxidative processes around necrotizing cells (Kato and Misawa, 1976) could lead to substantial inactivation of catalase by accumulation of inactive enzyme intermediates. Under conditions of such oxidative stress, SA might help to maintain and/or reestablish a basal level of catalase activity. These protective, antioxidative properties of SA might serve to limit the impact of the oxidative processes associated with development and spread of the lesion.

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**REFERENCES**

Abri, H. (1984) Methods Enzymol. 105, 121–126

Bi, Y.M., Kenton, M., Lur, D., Darby, R., and Draper, J. (1995) Plant J. 8, 235–245

Bonagura, C.A., Sundaramoorthy, M., Pappa, H. S., Patterson, W. R., and Foulou, G. T. (1996) Biochemistry 35, 6157–6165

Chen, Z., Ricigliano, J. W., and Klessig, D. F. (1993a) Proc. Natl. Acad. Sci. U. S. A. 90, 9533–9537

Chen, Z., Silva, W., and Klessig, D. F. (1993b) Science 262, 1883–1886

Chen, Z., Malamy, J., Hennig, J., Conrath, U., Sanchez-Casas, P., Ricigliano, J., Silva, H., and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4134–4137

Conrath, U., Chen, Z., Ricigliano, J. W., and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7143–7147

Dangl, J. L. (1995) Cell 80, 363–366

Deisseroth, A., and Dounce, A. L. (1970) Physiol. Rev. 50, 319–375

Dempsey, D. A., Dennis, B., and Smith, W. G. (1988) Arch. Biochem. Biophys. 230, 129–134

Dempsey, D. A., and Klessig, D. F. (1995) Bull. Inst. Pasteur 93, 167–186

Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryals, J. A., and Dangl, J. L. (1994) Cell 77, 565–577

Doke, N., and Ohashi, Y. (1988) Physiol. Mol. Plant Pathol. 32, 163–175

Durner, J., and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11312–11316

Enyedi, A., Yalpani, N., Silverman, P., and Raskin, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2480–2484

Escobar, J. A., Babus, M. A., and Lissi, E. A. (1996) Free Radical Biol. & Med. 20,
