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Identification of a soluble salicylic acid-binding protein that may function in signal transduction in the plant disease-resistance response

(systemic acquired resistance/plant defense mechanism/spin-column exclusion chromatography/ligand–protein interaction)

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ABSTRACT **It has recently been demonstrated that salicylic acid (SA) may serve as an endogenous signal molecule in the induction of systemic acquired resistance in tobacco and cucumber. In addition, SA is an endogenous regulator of heat and odor production in the inflorescence of some thermogenic plants. No information, however, is currently available concerning the mode(s) of action of SA in plant signal transduction. In a search for possible cellular factors that directly interact with SA, we have detected and partially characterized a SA-binding protein in tobacco leaves. The SA-binding activity is both SDS and proteinase sensitive and behaves as a soluble protein with an apparent mass of 650 kDa. The protein has an apparent K_d of 14 μM for SA, which is consistent with the range of physiological concentrations of SA observed for the induction of plant resistance responses. Furthermore, the ability of SA analogues to compete with SA for binding to this soluble protein is strictly correlated with their biological activity to induce the expression of genes associated with disease resistance. Biologically active analogues effectively inhibit SA binding while biologically inactive analogues do not. These results collectively indicate that this SA-binding protein may play a role in perceiving and transducing the SA signal to appropriate response elements, which ultimately activate one or more of the plant disease-resistance responses.**

Infection of plants with pathogens can activate a number of plant defense mechanisms against pathogen proliferation and secondary infection (1, 2). These induced defense mechanisms are assumed to result from the activated expression of a number of "defense-related" genes (3), whose products include enzymes involved in phenylpropanoid metabolism (4), proteinase inhibitors (5), cell wall proteins (6), peroxidases (3), hydrolytic enzymes (7), and the pathogenesis-related (PR) proteins (8, 9). In addition to the local responses at or near the sites of infection, many of these defense mechanisms are often activated in other parts of the plant to establish systemic acquired resistance, wherein the plant exhibits enhanced resistance to subsequent challenges by the same or even different pathogens. At present, little is known concerning the signals and transduction mechanisms involved in the establishment of resistance to the primary infection or of systemic acquired resistance.

Recently, Malamy *et al.* (10) reported that the endogenous salicylic acid (SA) levels in tobacco mosaic virus-resistant, but not susceptible, cultivars of tobacco increase at least 20-fold in inoculated leaves. SA also increased 5- to 10-fold in uninoculated leaves of tobacco mosaic virus-infected, resistant plants. This increase in SA levels paralleled the induction of PR gene expression. Metraux *et al.* (11) observed that the concentration of SA in phloem sap of cucumber plants increased transiently just prior to the establishment of systemic acquired resistance by inoculation with either tobacco necrosis virus or the fungal pathogen *Colletotrichum lagenarium*. Thus SA, a known exogenous inducer of several PR genes and disease resistance (for review, see ref. 9), appears to be an endogenous signal molecule in the induction pathway for disease resistance. In addition, it has been previously demonstrated that SA functions as an endogenous regulator of heat production in the inflorescence of some thermogenic lilies (12). Since exogenous SA has been found to influence several other physiological and biochemical processes of plants, including biosynthesis of the plant hormone ethylene (13), stomatal closure (14), and ion uptake (15), SA may play an important function in several other signal transduction pathways.

While studies on the roles of SA in various aspects of plant signal transduction should provide insights into the molecular mechanisms by which plants respond to environmental stresses, we are particularly interested in the role of SA in the induction of the disease-resistance response. As one approach to identify cellular elements that mediate induction of the resistance response by SA, we have partially characterized a soluble SA-binding protein in tobacco leaves. Several lines of evidence, including binding affinity and specificity, suggested that the SA-binding protein may be involved in perceiving and transducing the SA signal in plant cells.

MATERIALS AND METHODS

Materials. [7-^{14}C]SA (55 Ci/mol; 1 Ci = 37 GBq) was obtained from New England Nuclear; SA and other benzoic acid derivatives were purchased from Sigma. Stock solutions (0.1 M) of these chemicals were made with deionized water and their pH was adjusted to 6.5 with KOH. Trypsin that had been treated with *p*-toluenesulfony-L-phenylalanine chloromethyl ketone was from Worthington. Pronase, a nonspecific protease isolated from *Streptomyces griseus*, was purchased from Calbiochem.

Preparation of Soluble Protein. Tobacco leaves (50 g) were sliced and homogenized with a Polytron in 4 vol of a homogenization medium containing 20 mM citrate (pH 6.5), 10 mM $MgSO_4$, and 0.6% polyvinylpyrrolidone. The homogenate, after filtration through four layers of cheesecloth, was centrifuged for 30 min at 40,000 × *g*, and the supernatant was recentrifuged for another 15 min at 40,000 × *g*. The resulting supernatant was brought to 45% of ammonium sulfate saturation. The precipitate was collected by centrifuging for 15 min at 10,000 × *g* and dissolved in 10 ml of a binding medium containing 10 mM citrate (pH 6.5) and 10 mM $MgSO_4$. The protein solution was then dialyzed against 4 liters of the same binding buffer overnight and then centrifuged for 15 min at

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Abbreviations: PR, pathogenesis related; SA, salicylic acid.
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10,000 × g to remove undissolved proteins prior to the binding assay. All the operations were carried out at 4°C. Protein concentrations were determined according to Bradford (16) with the Bio-Rad protein assay kit.

[^{14}C]SA Binding. For a typical binding assay, 15 mg of soluble protein in a final vol of 1 ml was incubated at 4°C for 2 hr in the presence of 10 mM citrate (pH 6.5), 10 mM $MgSO_4$, and 5 μM [^{14}C]SA (55 Ci/mol). Bound and free [^{14}C]SA were then separated by a spin-column exclusion chromatography method similar to that described by Penefsky (17). Briefly, a 1.5-ml Eppendorf tube was punctured at the tip with a 20-gauge needle and a small amount of glass wool was added to cover the small hole. The tube was then filled with Bio-Gel P-6DG desalting gel (exclusion limit, 6 kDa) (purchased from Bio-Rad), which had been equilibrated with the binding buffer described above. Excess liquid in the gel was removed by centrifugation for 5 min at full speed in a Dyanic centrifuge (Becton Dickinson). The procedure was then repeated several times until the gel filled the tube. After incubation with [^{14}C]SA, 150 μl of protein binding sample was loaded onto the tube column, which was then rapidly centrifuged for 2 min and the solution of proteins and bound [^{14}C]SA that was excluded from the gel was collected into another tube that had been placed under the tube column during the centrifugation. One hundred microliters of this solution was then used to determine the amount of bound SA. All determinations were done in triplicate unless otherwise stated. Background radioactivity, due to the run-through of a trace amount of free [^{14}C]SA, was determined with 5 μM [^{14}C]SA in the absence of protein. Nonspecific binding was determined in the presence of 1 mM SA. K_d values were determined by Scatchard analysis with [^{14}C]SA concentrations ranging from 2 to 70 μM.

Molecular Mass Estimation by Gel Chromatography. To estimate the molecular mass of the SA-binding protein, the crude soluble extract (15 mg/ml) from a narrow ammonium sulfate cut (30–40%) was fractionated on a Sephacryl S-300 Superfine (Pharmacia) gel filtration column (1.5 × 100 cm). The sample in a 1-ml vol was eluted with the same binding buffer at a flow rate of 8.5 ml/hr at 4°C. The fractions (0.85 ml) were assayed for SA-binding activity as described above except with a higher concentration (15 μM) of [^{14}C]SA. The elution volumes (V_e) of six molecular mass standards, as well as the bed volume (V_b) and void volume (V_o) of the column, were determined under the same conditions and a calibration curve was obtained by plotting the K_{av} value [$(V_e - V_o)/(V_b - V_o)$] against the logarithm of the molecular mass of each standard.

RESULTS

Spin-Column Exclusion Chromatography Method for Identification of Binding Factors. The basal level of SA in uninfected tobacco leaves is ≈0.2 μM and can be induced to 2–15 μM by tobacco mosaic virus infection (10). This suggests that a SA-binding protein that perceives the SA signal should have a relatively high K_d for SA compared to hormone-binding receptor proteins (typically with K_d < 10 nM). Furthermore, the concentration of the binding factor is likely to be very low in the crude extracts used in the initial characterization of the binding activity. These two facts necessitated the application of a very sensitive and reliable assay method to identify the SA-binding factor(s). Indeed, when we first used an equilibrium dialysis method, no SA-binding activity was detected in the soluble fraction prepared from tobacco leaves (data not shown). However, when spin-column exclusion chromatography (17) was used to separate the bound [^{14}C]SA (excluded) from the bulk free SA (included), binding activity for [^{14}C]SA was detected in the soluble fractions (Table 1). Trace amounts of free [^{14}C]SA were found in the void volume of the spin column, but this composed only ≈0.2% of the total [^{14}C]SA loaded on the column and was <15% of the total radioactivity present in the void volume under our standard assay conditions (Table 1).

Table 1. Binding of [^{14}C]SA by soluble proteins of tobacco leaves

	[^{14}C]SA bound, dpm per 100 μl	
Assay condition	Protein from 0–45% $(NH_4)_2SO_4$ cut	Protein from 45–80% $(NH_4)_2SO_4$ cut
5 μM [^{14}C]SA	1129 ± 64*	259 ± 21
5 μM [^{14}C]SA plus 1 mM SA†	167 ± 11	156 ± 20
5 μM [^{14}C]SA plus 0.5% SDS	182 ± 14	135 ± 8
5 μM [^{14}C]SA without protein	132 ± 12	

Values were obtained from three independent binding assays and are reported with the sample (n = 3) SD. Each 100 μl of binding mixture contains 1.5 mg of proteins and 60,000 dpm of [^{14}C]SA.

*When the excluded protein–SA complex was rechromatographed, ≈70% remained intact. This is consistent with the time required for rechromatography (≈5 min) and the dissociation kinetics shown in Fig. 1.

†Unlabeled SA and [^{14}C]SA were added simultaneously. Similar results were obtained when excess unlabeled SA was added 30 min prior to [^{14}C]SA.

SA Binding. Most of the SA-binding activity in the soluble fraction prepared from tobacco leaves could be precipitated by 45% ammonium sulfate saturation (Table 1). In the presence of excess unlabeled SA, binding of [^{14}C]SA was dramatically inhibited, indicating that most of the binding activity for [^{14}C]SA is specific (saturable). SA binding reached equilibrium in 90–120 min at 4°C (Fig. 1). This binding was reversible since excess (1 mM) SA added to the protein solution preincubated with [^{14}C]SA displaced the radiolabeled ligand with a half-life of ≈40 min (Fig. 1). Scatchard analysis showed a single class of binding activity with a K_d of 14 μM and a B_{max} of 5 pmol per mg of protein (Fig. 2). Since there might be some dissociation of bound [^{14}C]SA during its separation from free SA by the spin-column method, the K_d value might be slightly overestimated, while the B_{max} might be underestimated. However, most of the solution containing bound [^{14}C]SA was separated from free SA during the first minute of centrifugation. Since dissociation (displacement) is relatively slow ($t_{1/2}$, 40 min) compared to the assay time (≈1

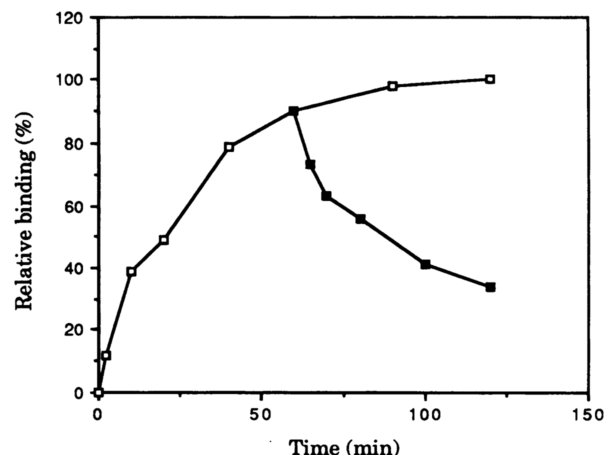

FIG. 1. Kinetics and reversibility of [^{14}C]SA binding. Binding of [^{14}C]SA (5 μM) approached equilibrium in 90–120 min at 4°C (□). This binding is reversible as shown by the addition of excess (1 mM) unlabeled SA after 1 hr of incubation (■).

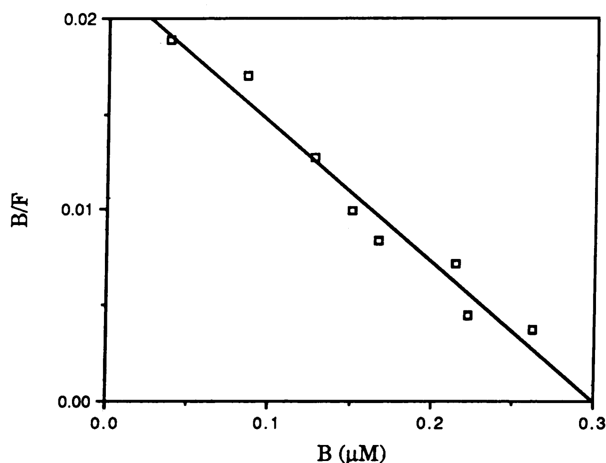

FIG. 2. Scatchard plot of SA binding. These experiments were performed on two independent protein preparations in quadruplicate with replicates varying by <10%. K_d was 14 μM, and B_{max} equaled 20 pmol per mg of soluble proteins from 0–45% ammonium sulfate. Since the proteins from this range of ammonium sulfate cut represented ≈25% of the total soluble proteins, the actual B_{max} was ≈5 pmol per mg of total soluble proteins. B, bound; F, free.

min), the deviation of the estimated K_d and B_{max} from their true values should be small.

Nature of the Binding Activity. Inclusion of 0.5% SDS in the reaction mixture reduced the binding of [^{14}C]SA to the background level (Table 1). Trypsin and Pronase, when preincubated with soluble fractions, significantly reduced subsequent binding of [^{14}C]SA (Fig. 3). Despite the difference in specificity, these two proteinases appeared to be equally effective in reducing the binding activity. At a relatively high concentration (50 μg/ml) of proteinases, >80% of the binding activity was destroyed after a 1-hr incubation at 30°C (Fig. 3). These results, together with the fact that the binding activity was nondialyzable and could be precipitated with ammonium sulfate, indicated that binding activity for [^{14}C]SA is a protein. When the soluble protein extract prepared from a 30–40% ammonium sulfate cut was chromatographed on a Sephacryl S-300 superfine gel filtration column, one symmetrical peak of SA-binding activity was found with a K_{av} value corresponding to an apparent molecular mass of ≈650 kDa (Fig. 4). SDS/PAGE of the fractions containing SA-binding activity indicated that the intensities of a number of protein bands correlated with SA-binding activities (data not shown). Since the soluble extract was prepared from a narrow, low ammonium sulfate cut (30–40%), the proteins in the extract may be relatively hydrophobic. Therefore, we cannot exclude the possibility that the SA-binding protein might be associated with other proteins in the extract through nonspecific hydrophobic interaction.

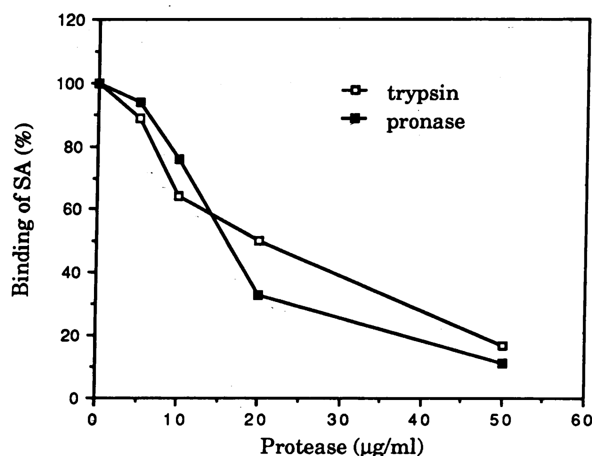

FIG. 3. Sensitivity of binding activity to proteinases. The soluble proteins (15 mg/ml) after ammonium sulfate cut (0–45%) were incubated with various concentrations of enzymes at 30°C for 1 hr, cooled on ice for 5 min, and assayed for [^{14}C]SA binding under standard conditions.

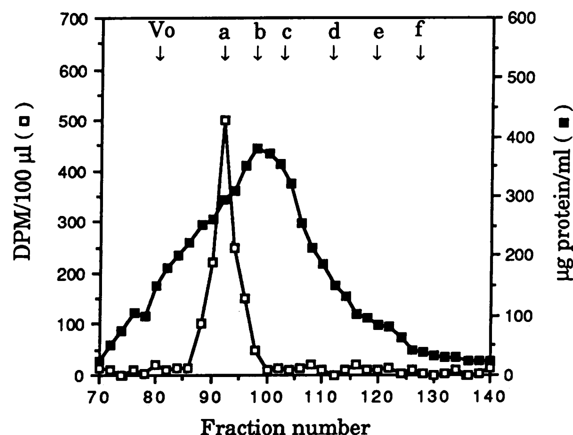

FIG. 4. Fractionation of the soluble extract by gel-filtration chromatography. The molecular mass of the SA-binding protein is estimated to be 650 kDa based on the elution volume of its peak of activity. The calibration of the Sephacryl S-300 column was obtained by a linear regression fit of a scatter plot of K_{av} versus logarithm of the mass of the following six proteins: a, thyroglobulin (669 kDa); b, ferritin (440 kDa); c, catalase (230 kDa); d, aldolase (158 kDa); e, albumin (67 kDa); f, ovalbumin (43 kDa). Void volume (V_o) was determined by using blue dextran. [^{14}C]SA binding was corrected for background radioactivity resulting from the run-through of a trace amount of free [^{14}C]SA.

Specificity of Binding. To assess the functional relevance of the SA-binding protein various SA analogues, with or without biological activity in inducing PR gene expression and plant disease resistance, were compared for their ability to compete with [^{14}C]SA for binding to the protein (Table 2). As expected, unlabeled SA competed effectively with [^{14}C]SA for the binding sites when added simultaneously. 2,6-Dihydroxybenzoic acid and acetylsalicylic acid (aspirin), which are active inducers of PR genes and resistance (18–20), were also both very effective competitors. Quantitatively, 2,6-dihydroxybenzoic acid competed as effectively as SA for the binding site(s), indicating that the binding site had similar affinities for these two compounds. Acetylsalicylic acid was a somewhat weaker competitor. In contrast, five benzoic acid derivatives with molecular structures similar to SA but that are unable to induce PR gene expression (18–20) were ineffective in inhibiting [^{14}C]SA binding to the protein under these conditions.

DISCUSSION

Spin-Column Exclusion Chromatography Method. There are several common methods available for quantitatively investigating the binding of small molecular mass ligands to soluble proteins, which include precipitation with ammonium sulfate, equilibrium chromatography, and equilibrium dialysis. Precipitation with ammonium sulfate provides a sensitive assay since it can effectively separate proteins with bound radiolabeled ligand from unbound ligand. However, others have reported artifacts generated by the precipitation assay in studying plant hormone binding (22), which could be attributed to changes in protein structures induced by high salt conditions. Equilibrium dialysis and equilibrium chromatography do not have this problem since they are carried out under physiological conditions. However, if the concen-

Table 2. Inhibition of [^{14}C]SA binding by benzoic acid derivatives

Benzoic acid derivative	2×		10×	
	dpm	Inhibition, %	dpm	Inhibition, %
Biologically active				
2-Hydroxybenzoic acid (SA)	1399 ± 50	52	345 ± 30	88
2,6-Dihydroxybenzoic acid	1406 ± 20	51	288 ± 25	90
Acetylsalicylic acid*	2100 ± 45	28	974 ± 28	67
Biologically inactive				
3-Hydroxybenzoic acid	2964 ± 200	0†	2947 ± 90	0†
4-Hydroxybenzoic acid	2914 ± 116	0†	2860 ± 86	1
2,3-Dihydroxybenzoic acid	2880 ± 150	1	2474 ± 45	15‡
2,4-Dihydroxybenzoic acid	2924 ± 159	0†	2878 ± 158	1
2,5-Dihydroxybenzoic acid	2961 ± 150	0†	2941 ± 192	0†

[^{14}C]SA (20 μM) binding was assayed in the presence of 40 μM (2×) or 200 μM (10×) unlabeled derivative. The dpm values listed represent specific binding (i.e., without including those dpm values resulting from nonspecific binding and run-through of free [^{14}C]SA). In the absence of unlabeled derivative, the dpm value for [^{14}C]SA (20 μM) binding is 2895 ± 139. Biological activity is based on White (18), Van Loon (19), and Abad *et al.* (20).

*In a complementary competition experiment, binding of labeled acetylsalicylic acid was inhibited by excess unlabeled SA.

†In these cases, 0% inhibition was given since addition of these unlabeled derivatives resulted in an apparent slight enhancement of [^{14}C]SA binding.

‡Doherty *et al.* (21) reported that in addition to SA, 2,6-dihydroxybenzoic acid, and acetylsalicylic acid, 2,3-dihydroxybenzoic acid was active in preventing induction of proteinase inhibitors by pectic fragments.

tration of binding protein is very low (such as in the crude total protein extracts) and the binding protein has a relatively high K_d (e.g., > 1 μM) for the ligand, the methods of equilibrium dialysis and equilibrium chromatography are impractical. Under these conditions, the ratio of bound to free ligand is essentially negligible. Therefore, one will observe, under these conditions, neither a significant difference in ligand concentrations between the two half cells (dialysis method) nor ligand-enriched peak fractions (conventional equilibrium chromatography). We report here the application of spin-column exclusion chromatography for studying SA binding. Unlike conventional chromatography or dialysis methods, spin-column exclusion chromatography allows the separation of the bound form of ligand from the bulk form of free ligand and increases the assay sensitivity. In contrast to the precipitation method, separation by spin-column chromatography is carried out under physiological conditions, and therefore the possibility of generating misleading binding artifacts during the separation is avoided. In our standard binding conditions, the ratio of bound [^{14}C]SA to free [^{14}C]SA is <0.02 even when a relatively high concentration of protein (15 mg/ml) is used to increase the binding activity. It would have been extremely difficult to detect this binding activity by common equilibrium dialysis or conventional chromatography. In addition to its high sensitivity, spin-column chromatography is very reproducible, convenient, rapid, and economical. Therefore, it provides an ideal way to study protein–ligand interactions.

Nature of the SA-Binding Protein. Since many receptors are integral membrane proteins, we initially assayed membrane fractions to detect SA-binding activity. However, no SA-binding activity was detected in the membrane fractions prepared from tobacco leaves by a standard precipitation method commonly used in studying membrane-bound receptor proteins (unpublished result). However, as described in this report, specific SA-binding activity was found in the soluble fractions. This binding factor is likely to be proteinaceous based on its sensitivity to proteinases and SDS, its precipitation by ammonium sulfate, and its nondialyzable nature.

The properties of this soluble SA-binding protein are consistent with its involvement in mediating a disease-resistance response transduced via SA. First, according to recent reports (10, 11), endogenous SA increases by 10- to 100-fold to levels of 2–15 μM at the onset of the plant resistance response after microbial attack. These concentrations of SA were determined based on the total fresh weight of plant materials. The local concentrations of SA might be considerably higher at or near the sites of infection. Any cellular factor that is responsible for mediating the primary biochemical response to SA must be able to sense this substantial change in the endogenous SA levels. The soluble SA-binding protein reported here satisfies this criterion. Its apparent affinity for SA (K_d = 14 μM) would allow it to respond to these alterations in SA levels by parallel changes in the ratios of the free form of the protein to the SA-bound form. The abundance of the SA-binding protein (≈5 pmol per mg of total soluble proteins) is higher than that reported for the putative glucan elicitor receptor (≈1.3 pmol per mg of membrane proteins) (23). This might be anticipated given the high levels of SA and the likely position of this signal within the transduction pathway leading to disease resistance. The levels of SA do not increase until 1–2 days after infection (10), presumably after the initial signal emitted from the plant–pathogen interaction has been transduced and amplified several times. This would account for the relatively high concentrations of the SA ligand (up to 15 μM). Efficient perception of this abundant signal molecule may require a relatively abundant binding protein. If the plant contained lower levels of SA-binding proteins, it might be less sensitive to changes in SA concentrations. A precedent for this is provided by studies on the auxin-binding protein. It was found that in both auxin-resistant and auxin-hypersensitive plants auxin sensitivity correlated with receptor abundance on the plasma membrane (for review, see ref. 24).

Second, the binding specificity of the SA-binding protein provides the strongest evidence implicating it in signal transduction, leading to disease resistance. This protein has binding affinity for only those SA analogues (2,6-dihydroxybenzoic acid and acetylsalicylic acid) that possess the biological activities of SA in induction of PR genes and disease resistance. In contrast, despite the similarity of their molecular structures to SA, those analogues that lack such biological activities fail to compete with SA in binding to this factor (Table 2). Thus, the specificity of binding for various SA

analogues is directly correlated with their biological activities in inducing PR genes and plant resistance.

Such a strong correlation would also argue against the possibility that this binding protein may be a SA-metabolizing enzyme. Moreover, the observed dissociation rate of bound [^{14}C]SA ($t_{1/2}$ = 40 min) (Fig. 1) is also unusually slow for a SA-metabolizing enzyme even at the relatively low temperature (4°C) under which the assays were conducted. Furthermore, we have observed that exogenous SA partially suppresses the modest increase of the SA-binding activity (2- to 3-fold) induced by an extended period of flotation of tobacco leaves on water. If the observed SA-binding protein was a SA-metabolizing enzyme, it is unlikely that the binding activity would be suppressed by the enzyme's own substrate. On the other hand, such a suppression of the induction of SA-binding activity by exogenous SA can be readily explained on the basis of a feedback mechanism if one assumes that the SA-binding protein is a factor that perceives and transduces the SA signal. Finally, we established that >90% of the labeled compound bound to the excluded proteins remained as SA, indicating the absence of significant SA-metabolizing activities in the binding mixture.

While it seems unlikely that the SA-binding protein described here is involved in SA metabolism, we must consider the possibility that it may be a SA-regulated cellular factor. This factor might be an enzyme or an enzyme complex, a DNA-binding protein, or an ion-channel component that may or may not be involved in propagating signal(s) in one or more pathways. If it is involved in signal transduction and the signal is part of the pathway(s) leading to disease resistance, then, as we suspect, the SA-binding protein may act as a receptor. On the other hand, it might play a role in transduction of a signal in another pathway. Alternatively, it might be the end target of SA, which, when associated with this ligand, exhibits altered properties/activities. To vigorously distinguish between these possibilities, purification and further characterization of the SA-binding protein are necessary. Such analysis should provide further insights into the mechanism(s) of action of SA-mediated physiological changes and perhaps the mode(s) of signal transduction in plants. Given the profound biochemical and physiological effects of SA (and aspirin) in animal systems and the many fundamental similarities between all organisms, these insights may have manifestations beyond the plant world.

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