Evidence against specific binding of salicylic acid to plant catalase

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Abstract **It was demonstrated that salicylic acid (SA) not only binds to catalase from differentiated higher plants and plant cell suspension cultures but also to those of fungi and animals. SA bound specifically to iron-containing enzymes, such as catalase, aconitase, lipoxidase and peroxidase, while not to iron-free plant enzymes. On the grounds of these experiments, the claim is further challenged that SA is a signalling compound and second messenger in plants that activates plant defense-related genes through elevated H_2O_2 levels by specifically inhibiting catalase activity. SA may just function as a phytoalexin.**

Key words: Salicylic acid; Unspecific binding to iron-containing proteins; Role in disease resistance

1. Introduction

In 1874 Kolbe, who discovered an industrially useful method to synthesize SA, called attention to its strong antimicrobial action [1]. SA in the following years became established as a very successful food preservative and continued to be used on an appreciable scale until the 1950's. SA and metabolites, such as glucosides, methyl esters, etc., are found in a considerable number of plant species (for review see [2]) and these facts point to the possibility that this antimicrobial acid serves as an endogenous plant defense compound, as it is known for a great number of other low molecular weight natural products [3]. Indeed, cucumber seedlings inoculated with the fungus *Colletotrichum lagenarium* or tobacco necrosis virus had higher levels of SA [4]. Also, inoculation of that plant with the phytopathogenic bacterium *Pseudomonas syringae* resulted in a drastic increase of SA concentration after a considerable lagtime (8 h) [5]. In search for possible cellular factors that directly interact with SA and might constitute a mode of action of SA in plant signal transduction, Klessig and coworkers [6,7] detected an SA-binding activity, characterized it as a salicylate inhibitable catalase [8], and showed its presence in a variety of plant species [9]. These authors [9] suggested that the mode of SA action in plant defense is to bind catalase and inhibit its activity. The resulting elevation of H_2O_2 (or other active oxygen species derived from H_2O_2) thus activates defense-related plant genes. This iron-containing catalase was addressed as a salicylate receptor [9], SA as a signal transducer [7] and an endogenous second messenger [9]. These experiments and the derived mode of action of salicylate drew considerable attention (e.g. [2,10,11]).

Analyzing Klessig's results [6–9], however, cast some doubts to his conclusions. First, in order to serve as a second messenger. SA should be freely translocatable in the plant. This, however, is not the case; this acid is immediately metabolically inactivated through conjugative mechanisms (for review see [2]). Second, endogenous concentrations necessary for catalase inhibition (10^{-3} M) are hardly ever reached within the plant. Third, the low specific activity of the [^{14}C]SA used [6] (55 Ci/mol) would never allow isolation of a true receptor. Fourth, the role of SA in binding to iron atoms contained in specific proteins was neither considered nor experimentally tested.

Considering these points, we set out to analyze the action of SA on iron-containing enzymes.

2. Materials and methods

2.1. Growth conditions of fungi and plant cell cultures

Aspergillus niger and *Neurospora crassa* were grown in a medium containing 1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, pH 5.0, at 30°C on a gyratory shaker at 120 rpm for 1 day. Plant cell cultures were grown in LS-medium [12] on a gyratory shaker (100 rpm) at 23°C and continuous light (650 lux) for 6 days.

2.2. Preparation of crude extracts

Plant cell cultures and fungi were harvested by suction on a Büchner funnel and deep frozen with liquid nitrogen. Crude extracts for testing the catalase in plant cell cultures were prepared by thawing the tissues with the triple amount of buffer (w/v) in 20 mM citrate buffer (5 mM $MgCl_2$, 1 mM EDTA), pH 6.5, under stirring on ice. The homogenate was filtered through 4 layers of cheese cloth and centrifuged for 30 min at 50,000 × *g*. The resulting supernatant was brought to 45% of ammonium sulphate saturation, centrifuged again at 50,000 × *g*, the pellet taken up in 10 mM citrate (10 mM $MgSO_4$), pH 6.5, and dialyzed against 5 litres of the same buffer overnight. Fungal crude extracts were prepared likewise except that the deep-frozen tissue was ground in an ice-cold mortar with a pestle and seasand. For testing aconitase, mitochondria of *Nicotiana plumbaginifolia* cell suspension cultures were prepared according to [13].

2.3. Binding assays

The [^{14}C]SA-binding test was performed according to Chen and Klessig [6]. After incubation, the assay mixture was transferred to a Sephadex G-25 column (1 × 10 cm) equilibrated with 20 mM citrate buffer, pH 6.5 (flow rate 0.5 ml/min). Fractions of 1 ml were collected and aliquots were measured for radioactivity in a scintillation counter (Berthold betaszint, BF 8000). The amount of protein was measured according to Bradford [14].

To determine the K_d values, the assays were treated by filtration centrifugation as described by Chen and Klessig [6].

2.4. Enzyme assays

Catalase: the catalase activity was assayed according to Chantrenne [15]. *Aconitase:* the assay system contained in a total volume of 1 ml, 100 mM Tris-HCl buffer, 0.1 mM citrate, 0.5 mM NADP, 2.5 nkat aconitase, and 2.5 nkat isocitrate dehydrogenase. The formation of NADPH was followed at 340 nm. *Lipoxidase:* the assay system contained in a total volume of 3 ml, 0.3 mM linoleic acid, 30 mM Tris-HCl buffer, pH 9.0, and 37 nkat lipoxidase. The reaction was followed photometrically at 234 nm (according to Sigma product information).

2.5. Materials

The following proteins were purchased from Sigma Co.: lipoxidase (*Glycine max*, 88,000 units/mg protein), aconitase (porcine heart, 6.9

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units/mg protein), peroxidase (horseradish, 200 units/mg protein) isocitrate dehydrogenase (porcine heart 32 units/mg protein), catalase (*Aspergillus niger*, 7080 units/mg protein), bromelain (*Ananas sativus*, 7.5 units/mg protein), ascorbate oxidase (*Cucurbita* species, 2000 units/mg protein), and amyloglucosidase (*Aspergillus niger*, 40 units/mg protein). Bovine serum albumin β-glucosidase (*Amygdalus dulcis* 20 units/mg protein), and alcohol dehydrogenase (*Saccharomyces cerevisiae*, 400 units/mg protein) were provided by Boehringer-Mannheim.

[7-^{14}C]SA (55 Ci/mol) was from New England Nuclear. SA and other phenolic compounds were from Sigma, Aldrich and Fluka. Sephadex G-25 was from Pharmacia.

3. Results

Repetition of the early binding studies showed that indeed proteins derived from *Nicotiana tabacum* tissue showed binding of SA of the same specific radioactiviy in same order of magnitude as previously reported [6] (Table 1). This salicylate binding protein was found in the 0–45% $(NH_4)_2SO_4$ fraction and to a much lesser extent in the 45–80% fraction (Table 1), exactly as published [9]. The binding was inhibited 89% by excess of unlabelled SA, indicating that bound [^{14}C]SA can be displaced from its site of attachment of the binding protein, as was shown [6–9]. In our studies, this binding protein was found also in microorganism-free cell suspension cultures of *Nicotiana plumbaginifolia* and, for instance, *Phaseolus radiatus* (Table 1). Klessig and coworkers [8] had reported earlier that this SA-binding protein was found to occur only in plants and plant-derived catalase but not by catalase derived from animal tissue, which indicated to them the specificity and regulatory response of the plant catalase. Repetition of their experiment showed that indeed bovine catalase does not bind [^{14}C]SA and no inhibition of its catalytic activity at 1 mM concentration was seen. However, at 25 mM or 100 mM SA concentration, bovine catalase is inhibited (14% and 40%, respectively). To further explore the specificity of this SA-binding effect, we turned to fungal extracts and observed good SA-binding activity in *Neurospora crassa* and *Aspergillus niger* (Table 1). Binding in these microorganisms occurred at the same magnitude as in dicotyledoneous higher plants (Table 1 and [9]). The occurrence of SA-binding proteins in fungi already cast doubt as to its role as a signal compound or a second messenger in higher plants, giving rise to the oxidative burst by inhibiting catalase [8]. Experiments were repeated with commercially available crystalline catalase from *Aspergillus niger* and binding of SA was again observed (Table 2). Clearly the heme-iron-containing catalase from a fungus bound SA to the same order of magnitude as higher plants and was inhibited as a consequence in its catalytic activity (88%). Since SA is known to be a bidentate ligand and can contribute to phenolate and carboxylate coordination with iron atoms [16], we considered testing, in addition to catalase, other iron-containing enzymes from plant and animal origin for their ability to bind [^{14}C]SA under the published conditions [9]. As shown in Table 2, the commercially available iron-containing enzymes,

Table 1
Binding of [^{14}C]SA by crude enzyme preparations from plant tissue and fungi

Protein	1 mM SA unlabelled	Specific activity (dpm/mg protein)	Binding relative to tobacco (%)
Nicotiana tabacum (leaves)	–	1922 ± 300	100
0–45% $(NH_4)_2SO_4$ cut	+	402 ± 50	
45–80% $(NH_4)_2SO_4$ cut	–	467 ± 55	
	+	365 ± 45	
Nicotiana tabacum	–	1929 ± 224	68
(cell suspension culture)	+	888 ± 156	
Nicotiana plumbaginifolia	–	2000 ± 257	83
(cell suspension culture)	+	743 ± 75	
Phaseolus radiatus	–	1770 ± 246	57
(cell suspension culture)	+	893 ± 157	
Glycine max	–	1495 ± 180	47
(cell suspension culture)	+	777 ± 77	
Aspergillus niger	–	1164 ± 80	58
	+	276 ± 60	
Neurospora crassa	–	1400 ± 146	62
	+	450 ± 65	

If not otherwise stated, the 0–45% $(NH_4)_2SO_4$ fraction [9] was taken for binding assays. The binding assay was performed according to Chen and Klessig [6].

Table 2
[^{14}C]SA-binding by soluble proteins of different origin and inhibition by unlabelled SA

Species	1 mM SA unlabelled	[^{14}C]SA bound (dpm/mg protein)	Enzyme inhibition by 1 mM SA (%)
Catalase (*Aspergillus niger*)	–	1436 ± 95	88
	+	95 ± 40	
Peroxidase (horseradish)	–	1159 ± 110	n.d.[a]
	+	220 ± 30	
Aconitase (*Nicotiana plumbaginifolia*)	–	2200 ± 250	78
	+	476 ± 50	
Aconitase (porcine heart)	–	33000 ± 600	46
	+	1317 ± 140	
Lipoxidase (*Glycine max*)	–	745 ± 70	9
	+	222 ± 25	
Amyloglucosidase* (*Aspergillus niger*)		0	n.d.
Bromelain* (*Ananas sativus*)		0	n.d.
Alcohol dehydrogenase* (*Saccharomyces cerevisiae*)		0	n.d.
Bovine serum albumin*		0	n.d.
β-Glucosidase* (*Amygdalus communis*)		0	0
Ascorbate oxidase* (*Cucurbita pepo*)		0	n.d.

The binding assays were performed according to Chen and Klessig [6]. The enzyme assays were performed as described in section 2.
*Non-iron-containing proteins.
[a]Due to reaction of the peroxidase substrates (e.g. 2,2′-azinobis(3-ethylbenzthiatoline sulfonic acid) (ABTS)) with salicylic acid, the inhibition of the reaction could not be determined.

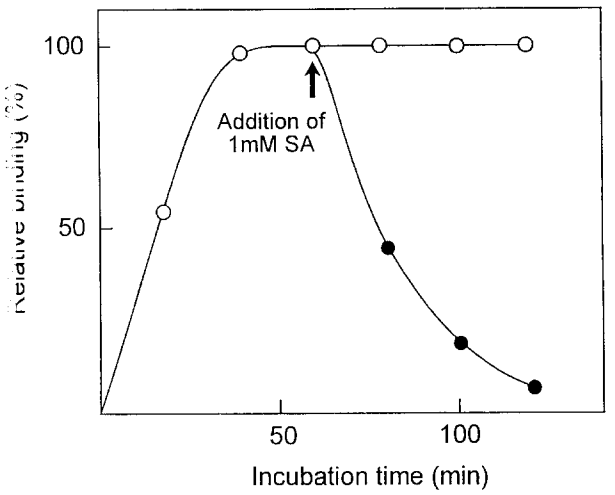

Fig. 1. Kinetics and reversibility of [^{14}C]SA-binding (5 μM). The binding assay using porcine aconitase was carried out according to [6]. Unlabelled SA (1 mM) was added at the time indicated to test the reversibility of the binding.

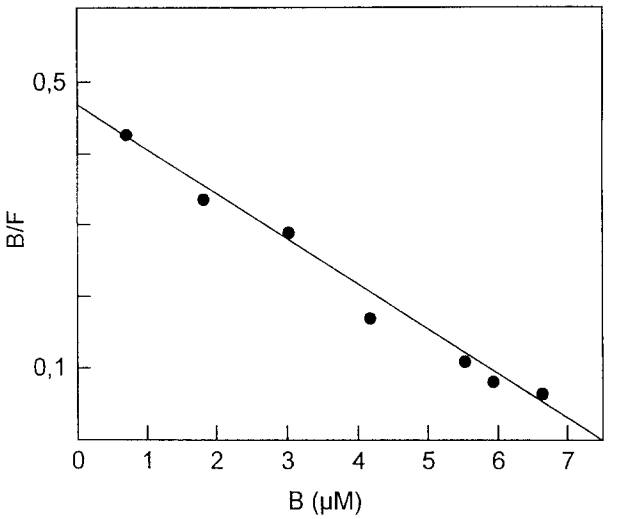

Fig. 2. Scatchard plot of SA-binding by porcine heart aconitase. The assay was performed according to [6]. K_d was 13.5 μM and B_{max} was 0.75 nmol/mg of protein. B = bound ligand; F = free ligand.

peroxidase from horseradish and lipoxidase from soybean, bound SA reversibly and through binding, impaired catalytic activity (Table 2). The iron-containing enzyme aconitase was prepared from mitochondria of *Nicotiana plumbaginifolia* [13] and found to bind SA at the highest level (Table 1) recorded in plants. The commercially available aconitase from porcine heart was, therefore, also tested and found to be an extraordinarily strong binding protein for SA. As much as 10% of the total labelled [^{14}C]SA supplied in the assay system was bound, which contrasts to 2% by the tobacco catalase [8]. As shown in Fig. 1, using porcine aconitase as binding protein, [^{14}C]SA was bound in a saturable way with first-order kinetics. The binding was reversible since excess unlabelled SA (1 mM) added to the protein solution preincubated with [^{14}C]SA displaced the radiolabelled phenol with a 'half-life' of 40 min. The kinetic of the displacement using the animal aconitase is in close agreement with the displacement curve obtained using the tobacco SA-binding catalase protein published previously [6], showing also a 'half-life' of 40 min. Five iron-free enzymes from plant and fungal origin that were tested for their ability to bind [^{14}C]SA were shown to be completely inert. Absolutely no binding or absorption of this bidentate phenolic acid was found to these metal-free proteins (Table 2).

Several SA analogues, hydroxybenzoic acids with varying numbers and positions of hydroxy groups, were used by Klessig and coworkers (e.g. [6–9]) to find a correlation between their ability to induce pathogen related gene expression, disease resistance in tobacco, and their ability to inhibit catalase activity from different plant species. Biologically active compounds were mainly SA, 2,6- and 2,3-dihydroxybenzoic acids, while all the other substituted benzoic acids were about 10-fold less active. This selectivity of the substitution pattern was again a major criterion to claim an important regulatory role in plant defense for SA [6,8]. In order to prove the specificity of catalase inhibition, fungal and tobacco catalase were compaired with regard to their sensitivities towards differently substituted benzoic acids. Table 3 shows a comparison between the homogenous tobacco catalase prepared by Klessig's group [8] and a crude 0–45% $(NH_4)_2SO_4$ cut, previously also used by Klessig and coworkers [6,9] but now prepared by us. There is quite a good correlation between the inhibitory properties of the individual hydroxybenzoic acids, even if there are some quantita-

Table 3
Inhibition of the catalytic activity of different catalase preparations by hydroxybenzoic acid derivatives

Inhibitor (1 mM)	Inhibition (%)			
	Aspergillus niger (Sigma)	*Aspergillus niger* (crude extract)	*Nicotiana tabacum* (crude extract)	*Nicotiana tabacum* (Chen et al. [8])
Salicylic acid	88	98	89	80
Acetylsalicylic acid	83	88	57	53
2,6-Dihydroxybenzoic acid	88	100	67	91
2,3-Dihydroxybenzoic acid	86	75	21	15
3,4-Dihydroxybenzoic acid	49	27	8	5
2,5-Dihydroxybenzoic acid	54	25	6	3
2,4-Dihydroxybenzoic acid	38	47	8	5
4-Hydroxybenzoic acid	52	17	2	4
3-Hydroxybenzoic acid	28	27	1	3

The enzyme activity was assayed according to Chantrenne [15].

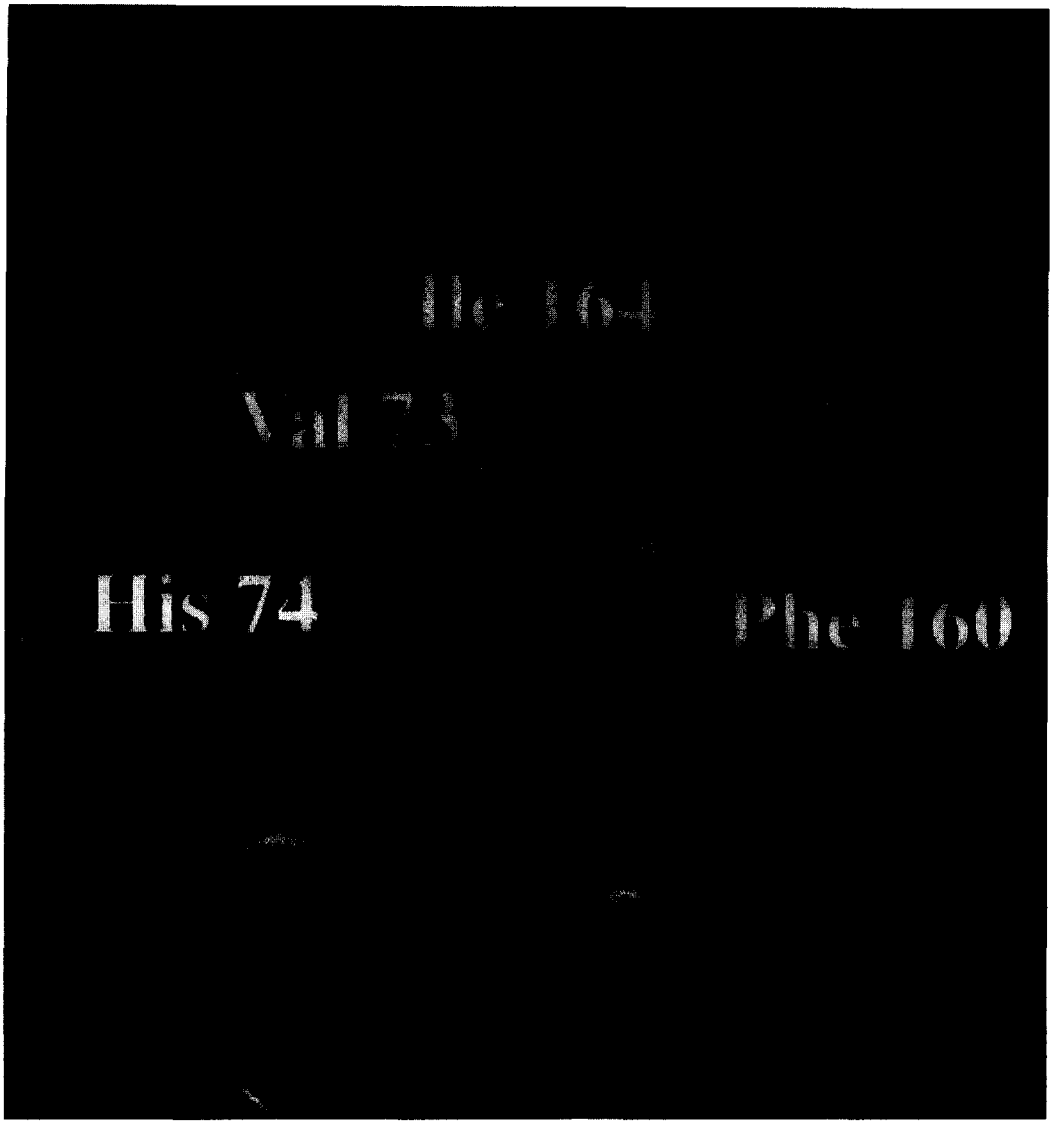

Fig. 3. A model of SA-binding to iron in the catalase heme group. The backbone structure of catalase is shown as a smoothed tube and selected side chains mentioned in the text are shown. Atoms that would come in steric conflict with SA are highlighted. Phe-160 can be rotated away from SA allowing some movement away from Val-73, but Ile-164 would have to adopt an unfavorable χ^1 torsion angle — in contrast to Val-164 which would be sterically acceptable.

tive differences. The fungal catalase of *Aspergillus niger,* however, also showed a similar inhibition pattern. Again, SA as well as 2,6- and 2,3-dihydroxybenzoic acids are dominant in their inhibitory action of the catalytic activity of the target enzyme while the rest of the substituted benzoic acids show considerable less inhibitory activity (Table 3). There was no binding specificity towards the plant catalase — the binding of SA to catalase of different plants, fungi and animals therefore has to be regarded as a general and unspecific property of this type of iron protein.

Scatchard analysis of the SA-binding to different plant catalases was used to demonstrate a single class of binding sites with an apparent K_d of 14–30 μM [6,9]. It is shown here (Fig. 2) that the iron-containing enzyme aconitase from pig heart also shows a single class of binding sites for SA with an apparent K_d of 13.5 μM when analyzed by the Scatchard computation.

To analyze these findings in a structural context, we constructed a model of SA, complexed to the catalytic heme-iron of bovine catalase. The structure of a 3-methyl salicylic acid–iron complex [16] was taken from the Cambridge Structural Database (Refcode JETTEH) and the iron atom superimposed on the iron atom of bovine catalase (PDB access code 8CAT) [17]. SA fits well into the catalytic cleft of the protein with only a single steric clash (Ile-164, see below) that may not be relieved by minor torsions around rotable bonds. A salt bridge may be formed with the catalytic histidine-74. All but one of the residues contacting SA are completely conserved among the 28 catalases currently in the SWISS-PROT sequence database. The only exception is Ile-164, which is valine in most plant enzymes. Interestingly, this substitution would relieve the severe steric conflict with C^δ of Ile-164 in the proposed mode of binding of SA (Fig. 3).

Aconitase binding and inhibition by SA may be modelled along the structure of *o*-hydroxybenzenethiolate [18] taken

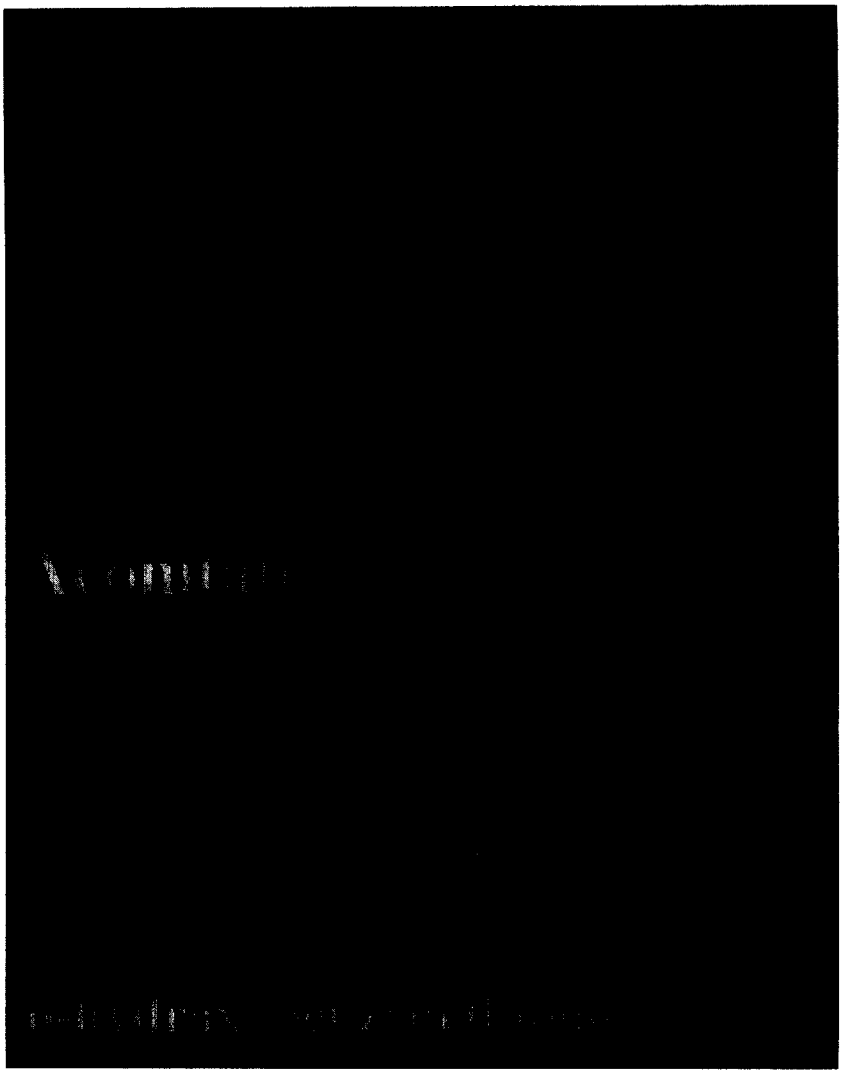

Fig. 4. A model of SA-binding to the [4Fe-4S] cluster of aconitase. The iron–sulfur cluster is shown with sulfur atoms colored light-gray. The three *o*-hydroxybenzene-thiolate groups liganded to the complex with their thiols can be perfectly superimposed on the liganding cysteine side chains of aconitase. This places the *o*-hydroxybenzenethiolate group that is bound to the cluster via both the thiol and the hydroxyl function exactly in the aconitase binding site. Comparison of the shaded Van der Waal's volumes of both compounds indicates that the binding site provides ample space for accomodation of SA or even substituted SA molecules.

from the Cambridge Structural Database (Refcode CAYGOY), which can be almost perfectly superimposed on the active site [4Fe-4S] cluster of bovine heart aconitase (PDB access code 1ACO) [19] placing the hydroxybenzenethiolate bound to Fe4 exactly into the aconitase binding site. Thus the binding of SA to aconitase poses no steric problems (Fig. 4).

4. Discussion

The concept developed by Klessig and coworkers (e.g. [20,21]) that the induction of systemic acquired resistance in plants is mediated by SA binding to catalase has attained considerable attention [2,10,11]. Inactivation by SA should elevate H_2O_2 levels in cells which in turn induces PR protein expression [9] This concept is based on a specific interaction between SA and a catalase isoform isolated and cloned from tobacco [6–9].

As shown in our contribution, SA binds to catalase present in different plant species, but also to catalase of fungal organisms [1,2] and at higher concentrations even to catalase from animals. Furthermore, it could be clearly demonstrated that SA-binding is a general property of iron-containing enzymes of plant and fungal origin. The binding of labelled SA to pig liver aconitase, an iron-binding enzyme, shows the same kinetic data and reversibility by unlabelled SA as previously demonstrated for catalase binding [6,9]. SA is known to have an extremely high stability constant for iron with a log K_1 = 16.48 in aqueous solution at 25°C [22].

Finally, both the stronger inhibition of plant catalases as compared to bovine catalase, as well as the intolerance to substitutions in positions 3, 4 and 5 but not 6, of the benzene ring, can be well rationalized on the base of the proposed binding model derived from small molecular data. The aconitase iron-sulfur cluster should be well accessible to SA and we predict that substitutions in all four available ring positions should be tolerated in this case.

We assume that the low specific activity of the commercially

available labelled SA and the abundance of catalase in plant tissue mislead Klessig and coworkers [6–9] to conclude that catalase is the only SA 'receptor' or sole binding component in the plant cell. Careful analysis of SA-binding to plant proteins using SA with a specific activity that is normally used in receptor-binding studies, would have shown that more iron-containing enzymes than catalase bind SA.

Most recently, independent experiments have been conducted showing also that the concept of elevated levels of H_2O_2 [9] as a consequence of possible catalase inhibition by SA is *not* required for systemic acquired resistance [23] and that H_2O_2 does not function downstream of SA in the induction of PR protein expression [24]. Furthermore, it has previously been shown that SA is essential for the development of systemic asquired resistance [25] but that the systemic acquired resistance inducing signal is not SA [26].

Based on these results and those presented herein, the role of SA to mediate plant defense by binding to and inhibiting catalase, and thereby increasing the concentration of H_2O_2 acting as second messenger in inducing plant defense gene expression [20,21], is now untenuable. We would predict that eventually it will be demonstrated that SA is induced as a result of pathogen attack in higher plants as a phytoalexin, a strongly antimicrobial agent, as has been known already for more than a hundred years [1].

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