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Benzoic acid 2-hydroxylase, a soluble oxygenase from tobacco, catalyzes salicylic acid biosynthesis

(*Nicotiana tabacum*/tobacco mosaic virus/cytochrome P450/acquired resistance)

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ABSTRACT **Benzoic acid 2-hydroxylase (BA2H) catalyzes the biosynthesis of salicylic acid from benzoic acid. The enzyme has been partially purified and characterized as a soluble protein of 160 kDa. High-efficiency *in vivo* labeling of salicylic acid with $^{18}O_2$ suggested that BA2H is an oxygenase that specifically hydroxylates the *ortho* position of benzoic acid. The enzyme was strongly induced by either tobacco mosaic virus inoculation or benzoic acid infiltration of tobacco leaves and it was inhibited by CO and other inhibitors of cytochrome P450 hydroxylases. The BA2H activity was immunodepleted by antibodies raised against SU2, a soluble cytochrome P450 from *Streptomyces griseolus*. The anti-SU2 antibodies immunoprecipitated a radiolabeled polypeptide of around 160 kDa from the soluble protein extracts of L-[^{35}S]-methionine-fed tobacco leaves. Purified BA2H showed CO-difference spectra with a maximum at 457 nm. These data suggest that BA2H belongs to a novel class of soluble, high molecular weight cytochrome P450 enzymes.**

Salicylic acid (SA), a phenolic compound ubiquitously distributed in plants (1), has been proposed as a likely endogenous signal in the induction of plant resistance to pathogens (2, 3). The observed increase in endogenous SA levels in pathogen-inoculated and uninoculated leaves was sufficient to induce systemic acquired resistance and synthesis of pathogenesis-related proteins (4). Work with transgenic tobacco plants expressing a bacterial gene encoding salicylate hydroxylase demonstrated that SA accumulation is required for the induction of systemic acquired resistance of tobacco against tobacco mosaic virus (5). However, grafting experiments suggested that, at least in tobacco, SA may not be a primary mobile signal in systemic acquired resistance (6).

In plants, SA is synthesized from trans-cinnamic acid by decarboxylation to benzoic acid (BA) and further 2-hydroxylation of BA to SA (7). The final step is catalyzed by benzoic acid 2-hydroxylase (BA2H), an enzyme that is constitutively expressed in tobacco but is highly induced by inoculation with tobacco mosaic virus (TMV) or application of BA (8). The induction results, in part, from enhanced *de novo* synthesis of BA2H protein. NAD(P)H or reduced methyl viologen can serve as *in vitro* reductants for BA2H.

Hydroxylation reactions are very important in the biosynthesis and metabolism of plant phenolic compounds. Most of these hydroxylations are catalyzed by cytochrome P450 monooxygenases (9). These enzymes form a superfamily of 50- to 60-kDa heme-containing proteins that have been extensively studied in animals and bacteria, but less well studied in plants. In eukaryotes, almost all known cytochrome P450s are microsomal proteins (10). In contrast to eukaryotic systems, most bacterial cytochrome P450 monooxygenases are soluble proteins (11–13). More than 200 P450 genes have been classified into subfamilies according to homology of their amino acid sequences (14). The homology between different forms of cytochrome P450 in eukaryotes and prokaryotes is very limited. However, some cases of antigenic crossreactivity between bacterial and plant cytochrome P450s have been reported (15).

Here we report the isolation and biochemical characterization of BA2H, an unusual, high molecular weight, soluble P450 oxygenase from tobacco which catalyzes the formation of SA from BA. This enzyme may play a key role in the activation of plant defenses against pathogens.

MATERIALS AND METHODS

Plant Culture and TMV Inoculation. Seeds of tobacco, *Nicotiana tabacum* L. (cv. Xanthi-nc, NN genotype), were sown and grown as described (4). The expanded leaves of 6- to 8-week-old plants were inoculated with 5 μg of the U1 strain of TMV or mock-inoculated without virus as indicated. The plants were incubated for 4 days at 32°C and then shifted to 24°C for up to 10 hr as described (7, 8). For experiments on *in vivo* inhibition of SA synthesis, tobacco leaves, attached to the plant, were syringe-infiltrated with 50 μM tetcyclacis in 5 mM potassium phosphate buffer (pH 5.5) or incubated inside the same transparent sealed chamber used for $^{18}O_2$ labeling experiments (see below), containing 0.5% or 5% (vol/vol) CO in purified air. The gas mixture was replaced every 24 hr for 3 days.

SA Labeling with $^{18}O_2$. A fully expanded tobacco leaf was inoculated with 5 μg of TMV. Twenty-four hours later, the inoculated leaf was placed in a transparent sealed chamber containing 20% $^{18}O_2$, 80% N_2, and 0.1% CO_2. A controlled flow of gases at 500 ml·min^{-1} through the leaf chamber was provided by an ASU (MF) air supply unit (Analytical Development, Kent, U.K.). Excess moisture was removed by passing the gases through a Drierite column. Temperature inside the leaf chamber was maintained at 22–24°C. Five days after inoculation, SA was extracted from the inoculated leaf, as previously described (4), methylated with ethereal diazomethane, and analyzed by HPLC and GC–MS. GC was performed with a DB-5MS capillary column (30 m × 0.32 mm, 0.25-μm film thickness; J. & W. Scientific, Rancho Cordova, CA) with a Varian 3400 gas chromatograph connected directly into the ion source of a Finnigan-Mat 8230 high-resolution double-focusing magnetic-sector mass spectrometer (Finnigan-Mat, San Jose, CA) via a heated transfer line maintained at 280°C (16). A Finnigan Mat SS 300 data system was used for data acquisition and processing.

Measurement of Phenolic Compounds and BA2H and BA4H Activities by HPLC. The phenolic content of tissue samples was determined after separation on a C_{18} reverse-phase HPLC

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Abbreviations: BA, benzoic acid; BA2H, benzoic acid 2-hydroxylase; SA, salicylic acid; TMV, tobacco mosaic virus.

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column and detection by on-line UV absorption and fluorescence detectors (7). BA2H activity was measured by using HPLC to monitor BA-to-SA conversion (8). The 4-hydroxylation of BA was quantified by the formation of *p*-hydroxybenzoic acid detected by UV absorption. Total SA (the sum of free and conjugated SA) was measured following chemical hydrolysis (base and acid) of leaf extracts (7, 8).

Preparation of Soluble and Microsomal Protein Fractions from Leaf Extracts. Leaves from plants inoculated with TMV and subjected to temperature shift for 10 hr were harvested, frozen in liquid nitrogen, and ground to a fine powder in a cold mortar. The pulverized tissue was suspended in 3 ml of 20 mM triethanolamine buffer, pH 7.4/14 mM 2-mercaptoethanol/1 mM phenylmethanesulfonyl fluoride (standard buffer) per g of tissue fresh weight. The suspension was mixed, filtered through four layers of cheesecloth, and centrifuged at 10,000 × *g* for 15 min. The resulting supernatant is referred to as crude extract. After centrifugation at 100,000 × *g* for 60 min, the supernatant contained the soluble proteins and the pellet contained microsomes.

Purification of BA2H. The soluble protein fraction of TMV-inoculated tobacco leaves was subjected to two sequential ammonium sulfate precipitations at 40% and 75% saturation. The 75% pellet was resuspended and desalted in a Sephadex G-25 column. After filtration through a 0.2-μm low-protein-binding filter, the proteins were loaded on a Mono Q FPLC ion-exchange column (Pharmacia). A Rainin FPLC/HPLC system (Rainin, Woburn, MA) controlled by Dynamax chromatography software was used. After sample injection, the column was washed isocratically for 12 min with 20 mM triethanolamine buffer (pH 7.3) at a flow rate of 1.5 ml·min^{-1}. The proteins were eluted with a 0–1.4 M KCl gradient (60 ml) in 20 mM triethanolamine buffer (pH 7.3) for 40 min and 1.5-ml fractions were analyzed for BA2H activity. The fractions eluted at 0.55 M KCl, containing maximum BA2H activity, were pooled, concentrated by ultrafiltration, and filtered through a 0.2-μm low-protein-binding filter. The sample was injected onto a Superose-12 FPLC gel filtration column and eluted at a flow rate of 0.75 ml·min^{-1} with 20 mM triethanolamine buffer (pH 7.3). Fractions (0.75 ml) were assayed for BA2H activity. Total protein elution profiles were monitored with an on-line ISCO AU-6 UV detector (ISCO) connected to the FPLC unit.

Immunoprecipitation of Soluble BA2H. The soluble fraction (1 ml) from tobacco leaf extracts was incubated with rabbit antibodies against cytochromes P450 from avocado or *Streptomyces griseolus* for 5 hr at 4°C. Thereafter, 40 μl of a protein A-agarose suspension [15 μl of beads in 25 μl of 10 mM Tris·HCl buffer, pH 7.4/150 mM NaCl/2 mM EDTA/0.2% (vol/vol) Nonidet P-40; buffer A] was added and the mixture was incubated overnight at 4°C with continuous rotation. Then the samples were centrifuged for 5 min at 10,000 × *g* and the supernatants were used to measure the remaining BA2H activity. The pellets were washed twice with 1 ml of buffer A, once with 1 ml of buffer A containing 500 mM NaCl, and once with 10 mM Tris·HCl buffer (pH 7.4). The washed beads were boiled for 10 min in SDS sample buffer and analyzed by SDS/PAGE.

Labeling of BA2H. Immediately before the temperature shift from 32°C to 24°C, the petiole of each excised mock- or TMV-inoculated tobacco leaf was immersed in 200 μl of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.5 mCi of L-[^{35}S]methionine (517 Ci·mol^{-1}; 1 Ci = 37 GBq). After solution was taken up (40–50 min), the leaf was fed twice with 200 μl of buffer and incubated under continuous light for 6 hr at 24°C. Thereafter, the leaf tissue was frozen in liquid nitrogen and extracts were prepared as described above.

***In Vitro* CO Treatment and Spectral Analysis.** CO was bubbled separately, in light or dark, through the BA2H reaction mixture containing the substrates without NADPH and through the crude soluble protein extract from temperature-shifted TMV-inoculated leaves. After 5 min of bubbling, the solutions were combined (3 volumes of reaction mixture and 1 volume of protein extract) and the reaction was started by the addition of 1 volume of NADPH solution. The final concentration of all components was as described (8). Mixtures were incubated for 30 min at 30°C in light or dark. CO was bubbled for 3 min through the sodium dithionite-reduced purified BA2H and the CO-difference absorption spectrum (400–550 nm) was recorded against the reduced BA2H solution as baseline in a Beckman DU 64 UV–visible spectrophotometer.

RESULTS

SA Biosynthesis Is Catalyzed by a CO/Tetcyclacis-Sensitive Oxygenase. GC–MS analysis of the methyl ester derivative of SA, isolated by HPLC from the TMV-inoculated tobacco leaf incubated in a sealed chamber initially containing 20% $^{18}O_2$, showed the presence of a molecular ion at *m/z* 154 (Fig. 1) that

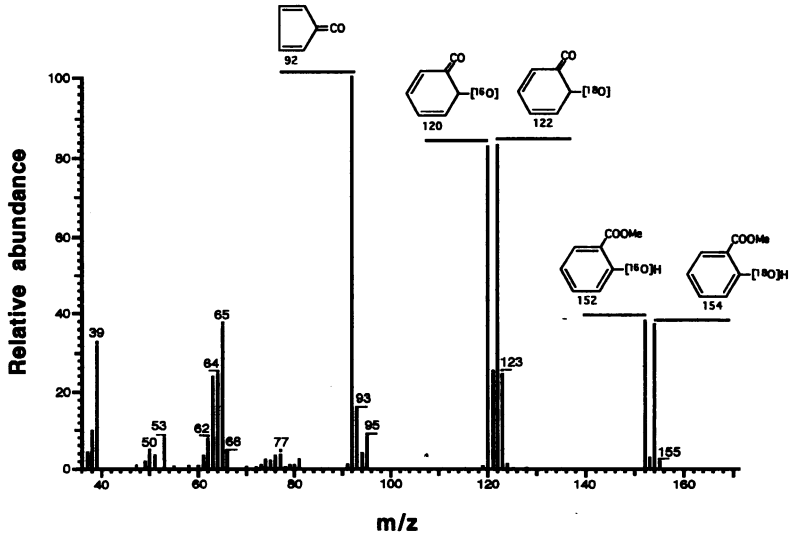

FIG. 1. Electron-impact mass spectral analysis of HPLC-purified $^{18}O_2$-labeled SA methyl ester from TMV-inoculated tobacco leaf. The numbers correspond to the mass of molecular ions and their fragments. The chemical structure of the molecular ions and fragments from unlabeled and $^{18}O_2$-labeled SA methyl ester are included.

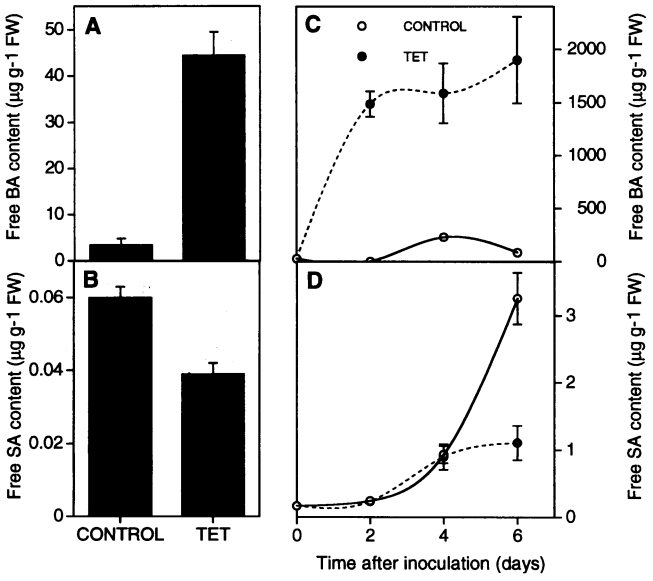

FIG. 2. Effect of tetcyclacis (TET) on the levels of free BA and SA in uninoculated and TMV-inoculated tobacco leaves. (*Left*) Free BA (*A*) and free SA (*B*) content in uninoculated healthy leaves. Leaves were infiltrated with 50 μM tetcyclacis and harvested 24 hr later. (*Right*) Time course of free BA (*C*) and free SA (*D*) accumulation in TMV-inoculated leaves. Leaves were inoculated with TMV at time 0 and syringe-infiltrated with 50 μM tetcyclacis twice a day. The mean values ± SE corresponding to three replicates are shown. A similar trend was observed when total phenolic contents were measured (data not shown). FW, fresh weight.

is shifted by 2 mass units compared with the molecular ion at m/z 152 of unlabeled methyl SA. This suggests that only one atom of heavy oxygen was incorporated into SA. The location of the heavy oxygen atom in the molecule was determined from the fragmentation pattern of methylated SA. The loss of a methanol group from SA methyl ester gives an ion at m/z 122 (Fig. 1) which is still shifted by 2 mass units compared with the ion at m/z 120 from fragmented unlabeled SA methyl ester (Fig. 1). This ion fragments further by elimination of two CO molecules. The elimination of the first CO produces an ion at m/z 92 which is not shifted in the spectrum of labeled SA methyl ester. Since it was shown, by using ^{14}C-labeled SA, that the first CO group is lost by a ring carbon (17), the loss of ^{18}O at this step suggests that a heavy oxygen atom was incorporated into the 2-hydroxyl group of SA. Utilization of molecular oxygen for the *in vivo ortho*-hydroxylation of BA is consistent with BA2H being an oxygenase.

CO inhibited the accumulation of SA in TMV-inoculated plants. Addition of 0.5% or 5% (vol/vol) CO to air for 3 days reduced SA content in TMV-inoculated leaves by 60% and 72%, respectively, when compared with an air control which

Table 1. Tetcyclacis blocks the induction of BA2H and the accumulation of SA

Treatment	BA2H activity, nmol·hr^{-1}·g^{-1} FW	Total SA content, μg·g^{-1} FW
Mock	1.23 ± 0.12	2.65 ± 0.31
TMV	8.53 ± 2.41	16.71 ± 2.65
TMV + TET	3.00 ± 0.85	2.03 ± 0.15

Tobacco Xanthi-nc (NN) leaves were inoculated TMV or with buffer (mock inoculation), incubated for 4 days at 32°C, and shifted to 24°C for 10 hr. TMV + TET corresponds to TMV-inoculated leaves that were treated with 50 μM tetcyclacis just before temperature shift. Values of BA2H activity and total SA content are the mean ± SE of four replicate samples. The experiment was repeated once with similar results. FW, fresh weight.

Table 2. Rates of 2- and 4-hydroxylation of benzoic acid with soluble and microsomal fractions of proteins from tobacco leaf extracts

Preparation	2-Hydroxylation, nmol·hr^{-1}	4-Hydroxylation, nmol·hr^{-1}
Crude extract	126.3 ± 18.6	—
Soluble fraction	72.6 ± 8.4	—
Microsomal fraction	0.6 ± 0.1	1046.7 ± 456.6

The crude extract (66 ml) was obtained from 20 g of tobacco leaves. Ultracentrifugation of crude extract resulted in the separation of the soluble (66 ml) and the microsomal (resuspended in 1 ml of standard buffer) fractions. Reaction mixtures contained 0.1 ml of the various protein fractions. Total 2- and 4-hydroxylating activities are expressed as the mean of triplicates ± SE. The experiment was repeated three times with similar results. —, Not detected.

averaged 2.27 ± 0.35 μg·g^{-1} fresh weight (n = 3). Tetcyclacis, a more specific norbornadiene inhibitor of cytochrome P450s, caused a 12-fold accumulation of free BA over the levels detected in control plants (Fig. 2*A*) and a parallel 35% decrease in the content of free SA (Fig. 2*B*) when infiltrated into healthy tobacco leaves at 50 μM concentration. Similarly, 2 days after TMV inoculation, the levels of free BA increased dramatically in tetcyclacis-infiltrated leaves compared with the untreated plants (Fig. 2*C*). High levels of BA persisted for at least 6 days after inoculation. Six days after inoculation, the free SA content of TMV-inoculated tetcyclacis-treated plants was 29% of that in untreated plants inoculated with TMV (Fig. 2*D*). Consistently, the treatment of inoculated leaves with tetcyclacis inhibited the induction of BA2H activity and the subsequent accumulation of SA to the levels similar to those detected in mock-inoculated plants (Table 1).

Soluble Nature and *in Vitro* Inhibition of BA2H from Tobacco Leaves. Almost all of the 2-hydroxylation activity of BA present in the crude extract was detected in the soluble protein fraction, whereas 4-hydroxylation was exclusively catalyzed by the microsomal fraction (Table 2). The microsomal fraction contained <0.5% of the total 2-hydroxylase activity present in the crude extracts. Detergents, such as Triton X-100 or 3-[(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS), did not release additional BA2H activity from the microsomal fraction (data not shown). Several inhibitors of cytochrome P450 hydroxylases inhibited *in vitro* the activity of the soluble BA2H. At 20 μM, paclobutrazol, tetcyclacis, tridiphane, and 1-aminobenzotriazole inhibited BA2H activity 100%, 88%, 78%, and 50%, respectively, com-

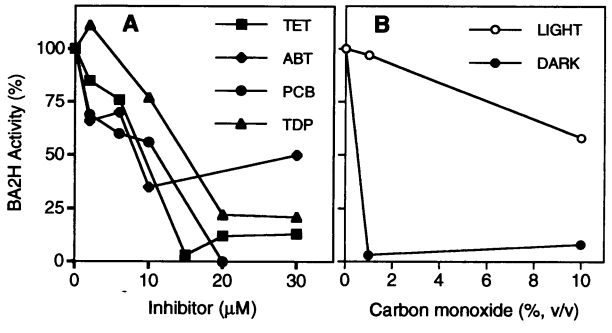

FIG. 3. *In vitro* inhibition of BA2H activity. (*A*) Effect of various concentrations of tetcyclacis (TET), 1-aminobenzotriazole (ABT), paclobutrazol (PCB), and tridiphane (TDP) on BA2H activity. Relative activities (%) were calculated as the mean of triplicates. The mean value ± SE corresponding to 100% activity was 2.12 ± 0.35 nmol·hr^{-1}·ml^{-1}. (*B*) Photoreversible inhibition of BA2H activity by CO treatment as described in the text. Relative activities (%) were calculated as the mean of triplicates. The mean values ± SE for 100% activity under continuous illumination and in the dark were 1.20 ± 0.58 and 0.71 ± 0.19 nmol·hr^{-1}·ml^{-1}, respectively.

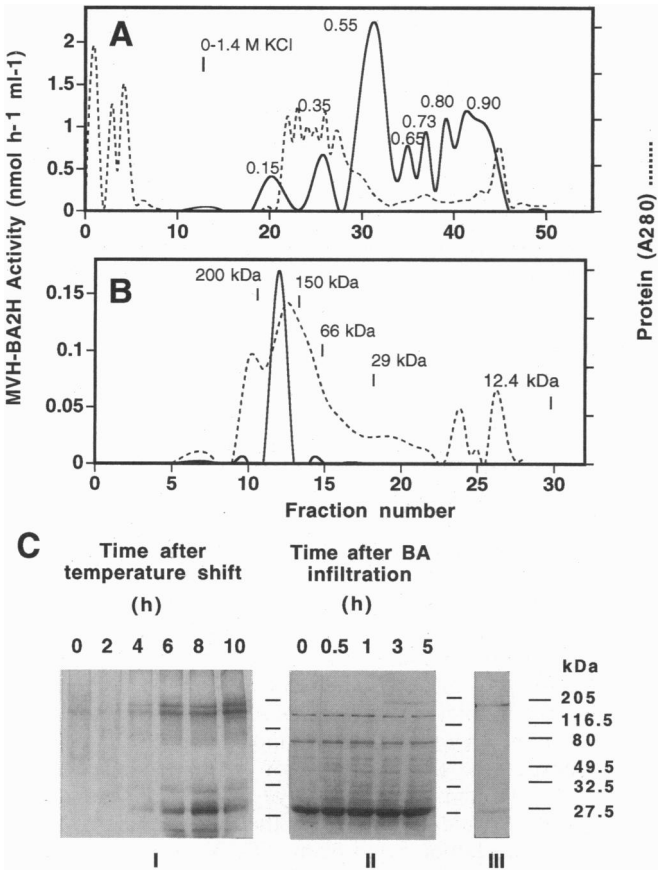

FIG. 4. Purification of BA2H from tobacco leaves. In the Mono Q ion-exchange (*A*) and Superose 12 gel filtration (*B*) elution profiles, the dotted line represents absorbance at 280 nm and the solid line, BA2H activity with reduced methylviologen (MVH). The beginning of the KCl gradient in *A* and the molecular size markers in *B* are indicated by vertical lines. The silver-stained SDS/10% polyacrylamide gels (*C*) correspond to soluble proteins from different times after temperature shift of TMV-inoculated tobacco plants (*T*), soluble proteins from different times after BA infiltration of healthy plants (II), and BA2H purified as described in the text (III).

pared with the activity in the absence of inhibitors (Fig. 3*A*). In addition, BA2H was inhibited *in vitro* by CO. Dark incubation of BA2H assay mixtures with either 1% or 10% (vol/vol) CO in air resulted in >90% inhibition of the activity present in the absence of CO (Fig. 3*B*). When the BA2H assay mixtures were incubated under the same conditions, but with continuous illumination, the inhibition was almost completely reversed for 1% CO. Approximately 50% reversal was detected for 10% CO (Fig. 3*B*).

Purification of BA2H from Tobacco Leaves. BA2H was partially purified from TMV-inoculated tobacco leaves by using a combination of ammonium sulfate fractionation, FPLC ion exchange on Mono Q, and gel filtration on Superose 12. The fractions containing the peak of BA2H activity eluted with 0.55 M KCl from Mono Q (Fig. 4*A*) were pooled, concentrated by ultrafiltration, and separated on a Superose 12 FPLC column. A single peak of activity corresponding to a protein of ≈160 kDa was detected (Fig. 4*B*). SDS/PAGE analysis of the purified enzyme showed two major protein bands of 160 kDa and 26 kDa (Fig. 4*C*, III). The 160-kDa band comigrated with that induced 3 hr after infiltration of uninoculated leaves with 0.1 mM BA (Fig. 4*C*, II) and also with one band induced 4 hr after temperature shift of TMV-inoculated plants (Fig. 4*C*, I).

Immunoprecipitation of BA2H. We tested the ability of antibodies against microsomal cytochrome P450 from ripened

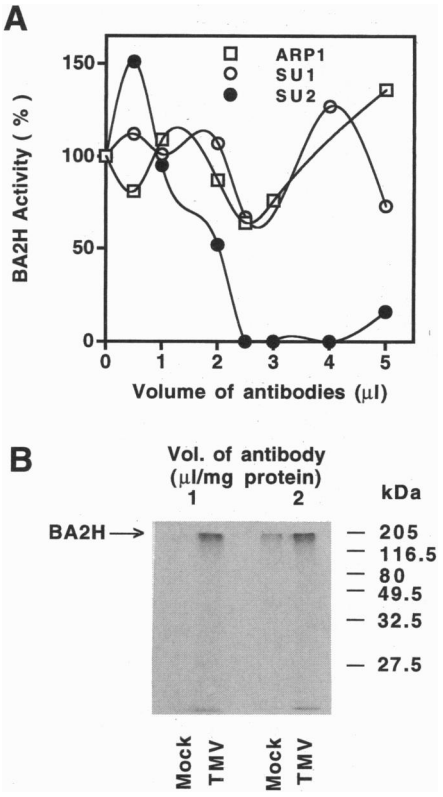

FIG. 5. (*A*) *In vitro* immunodepletion of BA2H activity. Antibodies against ARP1 cytochrome P450 from avocado, SU1 cytochrome P450 from *S. griseolus*, and SU2 cytochrome P450 from *S. griseolus* were used at a 1:500 dilution. Values of BA2H activity (%) were calculated as the mean of triplicates. The mean value ± SE corresponding to 100% activity was 1.99 ± 0.44 nmol·hr^{-1}·ml^{-1}. (*B*) Immunoprecipitation of ^{35}S-labeled BA2H from TMV-inoculated tobacco leaves. Mock- and TMV-inoculated tobacco leaves were labeled with L-[^{35}S]methionine for 6 hr after temperature shift from 32°C to 24°C, and extracts were subjected to immunoprecipitations with anti-SU2.

avocado (ARP1) or against two immunologically distinct soluble cytochrome P450s (SU1 and SU2) from *Streptomyces griseolus* to immunodeplete BA2H activity (Fig. 5*A*). Neither anti-ARP1 nor anti-SU1 antibodies immunodepleted the ac-

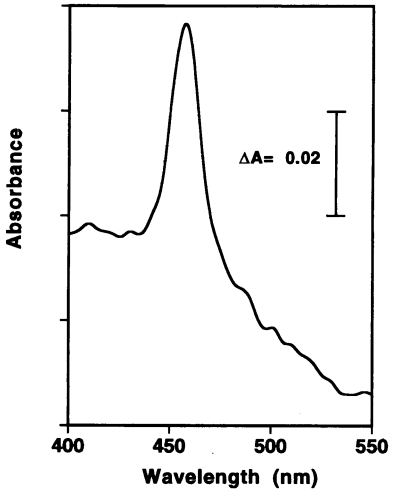

FIG. 6. CO-difference spectrum of partially purified BA2H. The absorption spectrum of protein (protein concentration, 8.3 µg·ml^{-1}; specific activity, 216 nmol·hr^{-1}·mg^{-1}) reduced by the addition of sodium dithionite prior to CO treatment was recorded against the reduced protein solution as baseline blank.

tivity significantly. However, 2 μl of 1:500-diluted antibodies against soluble SU2 completely immunodepleted the soluble BA2H activity. Incubation of mock- and TMV-inoculated tobacco leaves with L-[^{35}S]methionine, and subsequent immunoprecipitation with anti-SU2, identified a radiolabeled protein of ≈160 kDa that was specifically induced in TMV-inoculated leaves (Fig. 5*B*).

CO-Difference Spectrum. Absorption spectral analysis of the purified enzyme after treatment with CO was performed. Fig. 6 shows the CO-difference absorption spectrum (400–550 nm) of sodium dithionite-reduced and CO-treated BA2H against the reduced BA2H protein solution as baseline. A peak of maximum absorption was detected at 457 nm. Although the peak was shifted by 7 nm when compared with the usual 450 nm maximum for other P450s, the BA2H absorption peak was specific for the CO-treated reduced BA2H. This peak was not detected when the untreated reduced protein was scanned against the oxidized protein solution (data not shown).

DISCUSSION

This paper describes the characterization of BA2H at the biochemical and molecular levels. Our data on the function of this enzyme in hydroxylating BA, coupled with its biochemical properties, suggest that BA2H is a soluble cytochrome P450 oxygenase, most likely a monooxygenase. We have demonstrated that BA2H acts as an oxygenase by *in vivo* labeling of the hydroxyl group of SA synthesized *de novo* with $^{18}O_2$ (Fig. 1). The enzyme was inhibited by standard cytochrome P450 inhibitors both *in vivo* and *in vitro*. The *in vivo* inhibition by tetcyclacis was accompanied by an accumulation of BA and decrease in SA when compared with the control untreated leaves (Fig. 2; Table 1). Tetcyclacis and other specific inhibitors of cytochrome P450s, such as paclobutrazol, tridiphane, and 1-aminobenzotriazole (17–20), inhibited BA2H activity *in vitro* at concentrations below 30 μM (Fig. 3*A*). In addition, the activity showed photoreversible inhibition by CO (Fig. 3*B*), a fundamental property of P450 enzymes. When reduced BA2H was treated with CO, a specific CO-difference absorption spectrum with a maximum at 457 nm was observed (Fig. 6). The 7-nm shift of the absorption maximum from the 450 nm characteristic of other P450s may be explained by slight differences in the structure of the BA2H chromophore and its molecular environment. For example, certain changes in the N-terminal amino acid sequence of the human P450 2D6 shift the maximum absorption for the CO-Fe^{2+} complex by as much as 6 nm (18).

BA2H specifically hydroxylates the *ortho* position of BA (Table 2). We have partially purified BA2H from TMV-inoculated leaves by using a combination of ion-exchange and gel filtration FPLC (Fig. 4). The purified enzyme solution contained mostly BA2H but the recovery of activity was low (<1% of the total activity present in crude extracts). The fact that both crude soluble protein extracts and the purified preparations of BA2H utilize NADPH or sodium dithionite-reduced methyl viologen as reductants (ref. 8 and data not shown) strongly supports our suggestion that BA2H is catalitically self-sufficient. A soluble high molecular weight cytochrome P450 monooxygenase from *Bacillus megaterium* (P450BM-3) is also a catalytically self-sufficient single protein (13, 19).

BA2H activity was immunodepleted by polyclonal antibodies raised against SU2, a soluble cytochrome P450 that is induced in *S. griseolus* by the herbicide chlorimuron ethyl (20) (Fig. 5*A*). This provides additional evidence that BA2H is related to other P450 proteins. In vivo labeling of plant tissues with L-[^{35}S]methionine followed by immunoprecipitation with anti-SU2 (Fig. 5*B*) and partial purification of the enzyme from TMV-inoculated leaves (Fig. 4) identified BA2H as a 160-kDa protein. Immunoprecipitation of BA2H by antibodies against soluble SU2 from *S. griseolus*, and not by microsomal cytochrome P450 from avocado, suggests that BA2H is more closely related to bacterial than to plant cytochromes P450. It is particularly interesting that tobacco BA2H is a soluble protein not associated with microsomes (Table 2). Most soluble cytochrome P450 enzymes characterized so far are of bacterial origin (11–13). To our knowledge, the only soluble eukaryotic cytochrome P450 has been identified in *Fusarium oxysporum* (21). Therefore, BA2H may be the first soluble cytochrome P450 identified in higher plant or animal systems. Interest in this enzyme is warranted because of its key role in the synthesis of SA, an important signal in plant–pathogen interactions. The isolation of the BA2H gene(s) from plants may provide a powerful tool to manipulate SA levels in plants, thereby altering resistance to pathogens.

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