Plant Polyketide Synthases Leading to Stilbenoids Have a Domain Catalyzing Malonyl-CoA:CO_2 Exchange, Malonyl-CoA Decarboxylation, and Covalent Enzyme Modification and a Site for Chain Lengthening†

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ABSTRACT: Stilbene synthases and the related bibenzyl synthases are plant polyketide synthases whose biological functions lie in the formation of antimicrobial phytoalexins. The formation of hydroxystilbenes from one molecule of acyl-CoA and three molecules of malonyl-CoA is catalyzed by a homodimeric 90 kDa protein and includes Claisen condensations and cleavage of a thioester followed by decarboxylation. Combining inhibitor studies, protein modifications, and site-directed mutagenesis, we were able to differentiate between the binding sites for malonyl-CoA and the regions responsible for the selection of the primer, *p*-coumaroyl-CoA or *m*-hydroxyphenylpropionyl-CoA, respectively. Mutations in the C-terminal part of the molecule or modification by photolabeling with *p*-azidocinnamoyl-CoA influence the overall reaction, the formation of hydroxystilbenes, but leave partial reactions, such as the malonyl-CoA:CO_2 exchange and the malonyl-CoA-dependent modification of the enzyme, unaffected. Data obtained with several kinds of stilbene synthase and mutant forms suggest that the malonyl-CoA-dependent covalent modification takes place at a cysteine residue in the N-terminal part of the enzyme. Mutations in the C-terminal half of the enzyme molecule do not interfere with the malonyl-CoA-dependent reactions.

Aldol reactions and the related Claisen condensations are mechanisms widely used in biochemistry for the formation of carbon−carbon bonds. Among the processes which rely on this kind of anabolism are the condensing enzymes of fatty acid biosynthesis and polyketide synthases leading to various natural compounds. Polyketides which are of interest because of their distinct physiological functions have been described from bacteria, fungi, and higher plants (Hopwood, 1990; Katz & Donadio, 1993; Hutchinson & Fujii, 1995). Stilbenes, a class of polyketides arising by chain lengthening of one molecule of a phenylpropane unit with three acetate units derived from malonyl-CoA, are restricted to a few plant genera (Hart, 1981; Kindl, 1985). At certain stages of development and upon elicitation by fungal signals, these plants synthesize hydroxystilbenes and their derivatives, which eventually function as fungistatic phytoalexins (Hain *et al.*, 1993; Fischer & Hain, 1994).

The biosynthetic sequence leading to the stilbene skeleton (Figure 1) includes (a) three consecutive Claisen reactions, which are succeeded by (b) an aldol reaction that forms the second aromatic ring, (c) the cleavage of the thioester bond, and (d) a decarboxylation of an intermediary stilbene carboxylic acid. It should be feasible to demonstrate the existence of the partial reactions and to find the corresponding domains in the amino acid sequence by homologies with enzymes catalyzing related processes. All of the reactions take place by the function of 90 kDa homodimeric enzymes, designated "stilbene synthases" (Schöppner & Kindl, 1984; Gehlert *et al.*, 1990). Structurally, stilbene synthases (Schröder *et al.*, 1988; Melchior & Kindl, 1991) are closely related to chalcone synthases (Schröder & Schröder, 1990) although the products and partial reactions are different. Even more similarities to the stilbene synthases were found with bibenzyl synthases (Reinecke & Kindl, 1994; Preisig-Müller *et al.*, 1995) which convert a phenylpropanoic acid derivative instead of a phenylpropenoic acid derivative and lead to dihydrostilbenes (bibenzyls).

By combining different enzymatic assays, protein modifications, and site-directed mutagenesis, we describe details of the enzymatic mechanisms of stilbene synthases and the related bibenzyl synthases. The investigation shows that malonyl-CoA is not only condensed with starter CoA esters but reacts also with the enzyme forming an acyl protein.

EXPERIMENTAL PROCEDURES

Isolation of Plant Enzymes and Preparation of Recombinant Enzymes. Stilbene synthases from peanut and grapevine were obtained from cell suspension cultures according to Schöppner and Kindl (1984) and Liswidowati *et al.* (1991) while the pine enzyme was prepared from young plants infected with *Botrytis cinerea* as described by Gehlert *et al.* (1990). For the preparation of the orchid bibenzyl synthase (Reinecke & Kindl, 1994) and the recombinant grapevine stilbene synthase forms, we used the bacterial expression of cDNA constructs in the vectors pDS (Melchior & Kindl, 1990) and *Escherichia coli* strain NM522 as host or pQE11 (Preisig-Müller *et al.*, 1995) and *E. coli* strain M15/pREP4 as host. Thus, the N-terminal region was either enlarged by the sequence MRIIRRP- preceding the original start methionine (Melchior & Kindl, 1990) or contained an additional stretch of 15 amino acid residues including a His-6-tag (Preisig-Müller *et al.*, 1995). For obtaining His-tagged mutant forms, pDS-M0 (pSV25; Melchior & Kindl, 1990) was cleaved with *Sac*I in the 3′-noncoding region. Following blunt end formation, the coding sequence was cut out near the start ATG of stilbene synthase using *Sal*I. The vector

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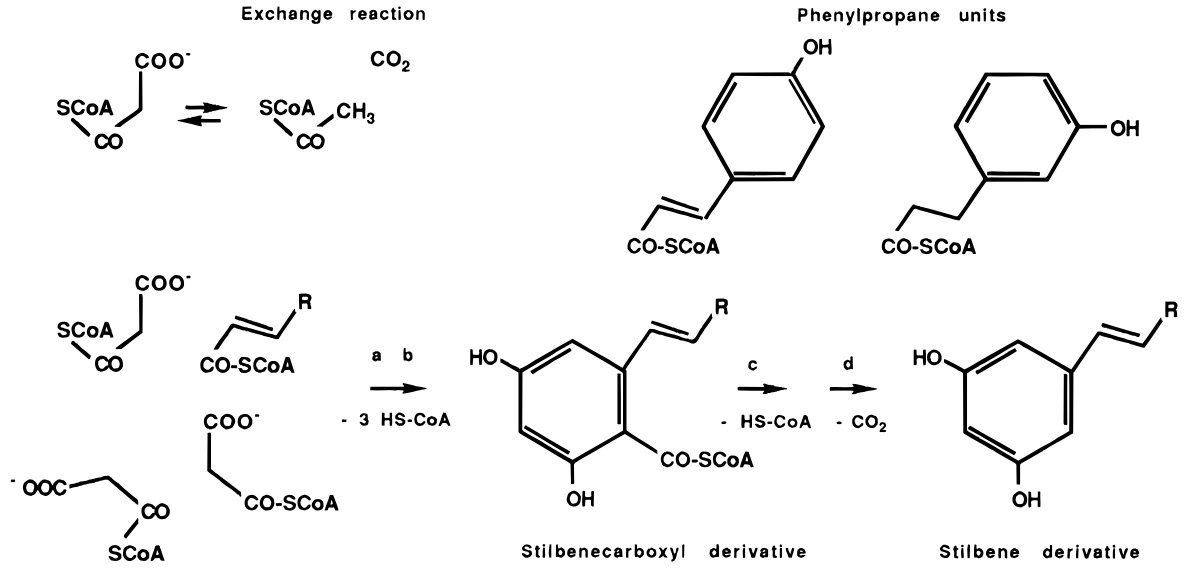

FIGURE 1: Chemical reactions involved in the formation of hydroxystilbene (resveratrol) from *p*-coumaroyl-CoA and malonyl-CoA. Partial reactions considered in this paper include a malonyl-CoA:CO_2 exchange reaction, repeated Claisen condensations of CoA ester originating from phenylpropane units (a), an aldol reaction (b) affording the cyclic compound, the thioesterase reaction (c) acting on a stilbene-2-carboxyl ester, and a decarboxylase step (d).

pQE11 was digested with *Hin*dIII. After blunt end formation, a *Sal*I restriction site was created. Basic recombinant DNA methods such as restriction enzyme digestions, ligations, transformations, and DNA sequencing were carried out as recommended by the suppliers of biochemicals and kits. Site-specific mutagenesis experiments were performed using the Transformer Site-Directed Mutagenesis Kit (Clontech) or the In Vitro Mutagenesis Kit (Amersham) and grapevine stilbene synthase M0 (Melchior & Kindl, 1990) as template.

A *Pvu*II site was introduced within the triplet coding for Ser-221 of bibenzyl synthase contained in pBibSy811 (Preisig-Müller *et al.*, 1995) to allow cleavage and subsequent fusion with the *Pvu*II site of stilbene synthase at the respective position. A second *Pvu*II site was present downstream of the multiple cloning site of pQE11. To construct plasmid pM12, we used M0 in pQE11 and replaced the region downstream of the *Pvu*II site with the respective fragment from pBibSy811. Equally, the plasmid pM13 was obtained by replacing the coding region of bibenzyl synthase downstream of the *Pvu*II site with the respective fragment taken from stilbene synthase. This procedure did not change the amino acid sequence in the range around Ser-221. All plasmids were partially sequenced to confirm the changes made.

A deletion removing a large part of the carboxyl terminus was designated pDS-M8 (129 amino acid residues removed). The construct pDS-M8 was generated by digesting pDS-M0 with *Ban*II which cuts at two sites in the C-terminal half of M0 cDNA. Religation of the shortened plasmid resulted in the M0 derivative M8, coding for a 29 kDa protein representing amino acid sequence 1−263 (related to wild type enzyme) and -KLKG at the C-terminus.

A rapid and efficient purification of the enzymes from cell suspension cultures or plants induced with fungal elicitor was achieved by absorbing the proteins of the crude extract on hydroxylapatite (Bio-Gel HTP) and removing all carbohydrates by washings. Subsequent to elution with phosphate, a combination of chromatography on Blue Sepharose-Cl-6B and Sephacryl S-200 led to almost pure enzymes. The M0 enzyme or its derivatives, recombinant enzymes derived from vector pDS, were obtained as apparently homogeneous proteins by binding the proteins present in a crude *E. coli* cell extract to hydroxylapatite in the presence of 20 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol. The enzyme eluted with a phosphate gradient buffer could be further purified by anion-exchange chromatography on a Mono Q FPLC column (Pharmacia). Throughout the purification procedures, it was advantageous to add 17% glycerol for stabilizing the enzyme activity. Molecular exclusion chromatography in the absence of glycerol led to a reduction of enzyme activity yield of more than 80%.

To purify His-tagged fusion proteins, *E. coli* strain M15-(pREP4) containing the respective pQE11 plasmid was grown at 35, 17, or 10 °C, respectively. Induction was achieved by adding IPTG to 1 mM. After 3 or 24 h (as indicated), the cells were harvested, resuspended in buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 2 mM mercaptoethanol), and lysed by freezing, thawing, and sonicating. The homogenate was clarified by centrifugation, and the supernatant was loaded onto Ni^{2+}-NTA agarose column (Preisig-Müller *et al.*, 1995). Bound proteins eluted with 120 mM imidazole in buffer A were finally recovered in 100 mM HEPES,[1] pH 8.0, and 2 mM mercaptoethanol. These conditions restored the enzyme activity.

Antisera were raised in rabbits against orchid bibenzyl synthase, grapevine stilbene synthase M0, and stilbene synthase prepared from peanut cell suspension cultures. Stilbene synthases were de novo synthesized in vivo by incubating bacterial strains or plant cell cultures with radioactively labeled precursors according to Liswidowati *et al.* (1991).

[1] Abbreviations: EDTA, disodium salt of ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-thiogalactopyranoside; PC, paper chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

Assays of Enzyme Activities. Stilbene synthase activity was determined by measuring the conversion of *p*-coumaroyl-CoA plus [2-^{14}C]malonyl-CoA (Amersham; 2.0 GBq/mmol) into resveratrol. The amount of radioactive resveratrol was quantified after TLC and liquid scintillation counting of the respective zone scraped off the thin-layer plate (Schöppner & Kindl, 1984). The test for malonyl-CoA:$^{14}CO_2$ exchange activity was carried out at pH 8.0 in a total volume of 100 μL by incubating the enzyme with 1.0 mM malonyl-CoA and 80 μM NaH$^{14}CO_3$ (adjusted to 200 MBq/mmol) at 30 °C for 60 min. Subsequently, the mixture was placed in an exsiccator along with NaOH, acidified, and left under reduced pressure. Radioactive malonic acid was purified by PC and quantified by liquid scintillation counting. For assaying the malonyl-CoA decarboxylase activity, [1,3-^{14}C]malonyl-CoA (NEN Du Pont; 1.2 GBq/mmol) was incubated with the enzyme in a two-arm minivessel containing the decarboxylase mixture in one side arm and 20% (v/v) phenylethylamine dissolved in methanol and spread on filter paper in the other side arm. After incubation for 6 min at 30 °C, 100 μL of phosphoric acid was added to the enzyme mixture via a rubber-sealed entry. The radioactivity of the CO_2 produced and absorbed on the filter paper was determined by liquid scintillation counting.

Malonyl-CoA was also used to demonstrate an acyl transfer to the enzyme: 100 pmol of enzyme was incubated with 1 nmol of [2-^{14}C]malonyl-CoA in 100 μL of 40 mM potassium phosphate, pH 8.0, at 30 °C for 30 min. Stilbene synthases chemically modified by the reaction with [2-^{14}C]-malonyl-CoA were purified by gel exclusion chromatography on Sephadex G-100 according to their molecular mass of 90 kDa. The radioactive proteins were run on PAGE and visualized by autoradiography or photoimaging.

For characterization of the radioactive acyl group transferred to the protein, the modified enzyme was treated with 0.2 M hydroxylamine at 37 °C for 50 min. The removal of the acyl group was demonstrated by rechromatography of the protein. In another experiment, the hydrolysis of the covalently labeled enzyme was carried out with 0.3 N sodium hydroxide at 30 °C for 60 min. Subsequently, the mixture was acidified, and the carboxylic acids were exhaustively extracted with ether. After addition of carrier compounds, acetic acid and malonic acid were characterized and quantified as *p*-bromophenacyl esters. As blank allowing for small amounts of acetic acid formed by chemical decarboxylation of malonic acid during the course of hydrolysis and purification we incubated stilbene synthase (5 μg) with 1 nmol of [2-^{14}C]malonic acid (10 kBq) and isolated the radioactive acetic acid as *p*-bromophenacyl ester as described above. This value (1.5%) was used to correct the yields of malonic acid and acetic acid obtained from acylated enzymes. Furthermore, to control the efficiency of the chemical hydrolysis of the radioactive modified enzyme, we determined the radioactivity remaining in the protein recovered by precipitation.

Inhibition Studies. *p*-Azidocinnamoyl-CoA was synthesized from *p*-azidobenzaldehyde (Bridges & Knowles, 1974) by condensation with malonic acid. ^{1}H-NMR ($CDCl_3$): 6.34 ppm (d, 15.9 Hz, 1 H); 7.00 ppm (d, 8.6 Hz, 2 H); 7.49 ppm (d, 8.6 Hz, 2 H); 7.68 ppm (d, 16.0 Hz, 2H). MS, *m/e* 189 (M^+, 77%); 161 (100%); 143 (99%); 115 (74%).

The reaction with *N*-hydroxysuccinimide led to the reactive ester which was further converted to the CoA ester according to Stöckigt and Zenk (1975). Data for *N*-hydroxysuccinimidyl *p*-azidocinnamate follow. ^{1}H-NMR ($CDCl_3$): 2.88 ppm (s, 4H); 6.54 ppm (d, 16.0 Hz, 1 H); 7.08 ppm (d, 7.9 Hz, 2 H); 7.55 ppm (d, 8.1 Hz, 2 H); 7.87 ppm (d, 16.0 Hz, 1 H). MS, *m/e* 286 (M^+, 40%); 258 (27%); 172 (97%); 160 (35%); 144 (97%); 132 (17%); 116 (100%); 89 (96%).

The CoA ester purified by PC showed three relative maxima in the absorption spectrum at 203 nm (ϵ = 20.8 cm^2/μmol), 260 nm (ϵ = 16.9 cm^2/μmol), and 336 nm (ϵ = 17.2 cm^2/μmol). Exposure of the CoA ester to UV light of 300−366 nm greatly reduced the absorption at 336 nm.

p-Azidocinnamoyl-CoA was used either as competitive inhibitor of the binding of *p*-coumaroyl-CoA or as a means for affinity labeling. In the latter case, the enzyme (0.1 μM) was incubated with the azido compound (0.1 mM), treated with UV light for 6 min, and subsequently purified by gel chromatography or PAGE.

Modification of stilbene synthases with *N*-ethylmaleimide was done by incubating the enzyme at pH 8.0 with 50 μM *N*-ethylmaleimide at 30 °C for 30 min. To ensure that the substrate binding thiol residues were fully reduced before reaction with thiol-specific inhibitors, protein pellets were resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 15% glycerol, 10 mM dithiothreitol, 2 mM benzamidine hydrochloride, and 2 mM EDTA. After incubation, the enzyme was exchanged, using a PD-10 gel filtration column (Pharmacia), into 100 mM potassium phosphate buffer, pH 7.5.

Inhibition of the stilbene formation was tested with diisopropyl fluorophosphate. The inhibitor (dissolved in isopropyl alcohol) was used at concentrations of 0.5−5 mM, and incubated with 25 ng of enzyme, 40 mM potassium phosphate, pH 8.8, 2% (v/v) ethylene glycol, 1.5 mM mercaptoethanol at 30 °C for 30 min. Cerulenin [(2*S*,3*R*)2,3-epoxy-4-oxo-7,10-dodecadienoylamide] was used as inhibitor of the condensation reaction at a concentration of 0.1 mM.

Loss of malonyl-CoA:$^{14}CO_2$ exchange activity was studied by treatment of stilbene synthase with mercurials such as *p*-hydroxymercuribenzoate (Subramani & Schachman, 1981). *p*-Chloro[^{203}Hg]mercuribenzoate was obtained by oxidation of tolyl[^{203}Hg]mercuric chloride in aqueous solution (Whitmore & Woodward, 1941). The conversion of 0.1 mmol of *p*-toluenesulfinate with 0.1 mmol of $^{203}HgCl_2$ (Amersham; adjusted to 300 MBq/mmol) was as according to Whitmore *et al.* (1941). *p*-Hydroxymercuribenzoate was purified by HPLC following the absorption at 233 nm (ϵ = 17 cm/μmol) (Boyer, 1954). 100 μg of purified M0 enzyme was incubated with 2 nmol of *p*-hydroxy[^{203}Hg]mercuribenzoate in a phosphate buffer, pH 8.0, at 30 °C for 30 min. Following purification of the protein by passage through a column filled with Sephadex G-50 (10 mL total volume) and concentration in a speed-vac, the labeled protein was subjected to exhaustive proteolysis by incubation with the protease Glu-C (Boehringer). A ^{203}Hg-labeled peptide was purified by HPLC on C_{18} reversed phase following the β-radiation, and the mercuribenzoate group was subsequently removed by addition of excess of dithiothreitol. After another HPLC chromatography, the N-terminal part of the peptide fragment was sequenced by automatic Edman sequencing.

Other Techniques. HPLC was performed with a C_{18} reversed phase column. Elution was with an gradient formed by 0.1% TFA and 80% aqueous acetonitrile, 0.07% TFA.

Table 1: Survey of the Wild Type Enzymes, the Mutants, and the Recombinant Proteins

enzyme form	derived from	construction	protein purification
arachis wild type			(Schöppner & Kindl, 1984)
grapevine wild type			(Liswidowati *et al*., 1991)
pine wild type			(Gehlert et al., 1990)
bibenzyl synthase	pBibSy811	*Hin*dIII fragment in pQE11	(Reinecke & Kindl, 1994; Preisig-Müller *et al.*, 1995)
M0	grapevine pSV25	pSV25 inserted into *Pst*I site of pDS12[a]	described in this publication
M1	M0	M0[b] in pQE11; C60A	Ni^{2+}-NTA affinity chromatography
M2	M0	M0 in pQE11; Δ(K53,K54,K55)	
M3	M0	M0 in pQE11; A37P	
M4a	M0	M0 in pDS12; S250A	
M4b	M0	M0 in pQE11; S250A	Ni^{2+}-NTA affinity chromatography
M5	M0	M0 in pQE11; N249D;A255D	HTP chromatography similar to M0
M6	M0	M0 in pQE11; C130A	
M7	M0	M0 in pDS12; C341A	
M8	M0	M0 in pQE11; Δ(264−392)	
M9[c]	M0	M0 in pQE11; C164A	Ni^{2+}-NTA affinity chromatography
M10[c]	M0	M0 in pQE11; VSI-(230−232)EAAD	Ni^{2+}-NTA affinity chromatography
M12[c]	M0, pBibSy811	M0(1−221) fused to bibenzyl synthase (222−392)	
M13[c]	M0, pBibSy811	bibenzyl synthase (1−221) fused to M0 (22−392)	

[a] Contains an N-terminal extension MRIIRRP (Melchior & Kindl, 1990). [b] *Sal*I fragment from M0 inserted into *Sal*I site of pQE11 resulting, compared to the wild type SV25, in an N-terminal extension, $MRGSH_6GIRRP$-. [c] Made by using kit and instructions provided by Clontech.

Electrophoreses, fluorography, and Western blots were performed according to Laemmli (1970) and Feussner and Kindl (1994). Protein content was determined according to Lowry *et al.* (1951). Limited proteolysis using V8 protease (Glu-C, Boehringer) was carried out as described earlier (Höhne *et al.*, 1996). Automated Edman degradation of peptides was performed using an Applied Biosystems model 477A sequencer.

RESULTS

Preparation and Characterization of Various Forms of Stilbene Synthases. Wild type stilbene synthases were prepared from cell suspension cultures of peanut or grapevine. Additional grapevine stilbene synthase forms were obtained either as fusion proteins slightly altered in the N-terminal region or as proteins with a 15 amino acid N-terminal extension carrying a His-tag. For the synthesis of bibenzyls (dihydrostilbenes), the His-tagged form of bibenzyl synthase from *Phalaenopsis* was used. The principle for constructing the vectors for site-directed mutagenesis and bacterial expression of fusion proteins and the data of the various forms of stilbene synthases used in this paper are summarized in Table 1.

For kinetic characterization, we concentrated on the properties of the fusion protein M0. This protein, identical to the wild type grapevine enzyme, except for a slightly altered N-terminal region possessing an extension MRIIRRP, exhibited K_m values slightly lower than the wild type enzyme: 1.2 μM for *p*-coumaroyl-CoA and 6.3 μM for malonyl-CoA instead of 1.6 μM for *p*-coumaroyl-CoA and 9 μM for malonyl-CoA; a v_{max} (related to μmol of resveratrol formed) of 350 pkat/mg of protein was calculated for M0 compared to 370 pkat/mg of the enzyme isolated from the plant.

To assess the extent to which the overexpressed proteins regain a configuration comparable to the wild type enzyme, we applied the knowledge that stilbene synthases possess sulfhydryl groups differently accessible to modification by Ellman's reagent or alkylation. The wild type enzyme was characterized by a single very reactive sulfhydryl group, and only after prolonged time periods of incubation with the sulfhydryl reagents, or by partially unfolding, were the other sulfhydryl groups modified. This property was utilized to assay whether the mutant forms behaved similarly and thus could be regarded as folded in a way similar to the wild type protein.

Stilbene Synthases Catalyze Malonyl-CoA:CO_2 Exchange, Malonyl-CoA Decarboxylation, Chain Lengthening of p-Coumaroyl-CoA, and Their Own Covalent Chemical Modification. As the studies were intended to unravel the presence and cooperation of two or more specific binding domains, we looked for possible partial reactions included in the multistep reaction sequence leading to the second aromatic ring (see Figure 1). Partial reactions have been found earlier with the related enzymes, namely, fatty acid synthase and chalcone synthase. By designing specific tests, it is possible to assay some partial activities of the stilbene synthases and to use the activities to characterize the total process.

Malonyl-CoA:CO_2 Exchange and Malonyl-CoA Decarboxylation. Using various forms of stilbene synthases (Figure 2), we observed malonyl-CoA:CO_2 exchange in the absence of the second substrate, i.e., *p*-coumaroyl-CoA. The exchange reaction characterized by a pH optimum of 8.0 obeyed hyperbolic kinetics. From the initial velocities, we calculated for the purified enzyme M0 a maximal velocity of the malonyl-CoA:CO_2 exchange reaction as 320 pkat/mg of protein. Thus, the maximal velocity of this partial activity (320 pkat/mg of protein) is similar to the velocity of the overall reaction leading to resveratrol (350 pkat/mg of protein). In partially purified preparations, the immunoprecipitation of the protein with antistilbene synthase antiserum caused a parallel decrease of both stilbene formation and malonyl-CoA:CO_2 exchange activity in the supernatant.

Methylmalonyl-CoA was tested as a potential substrate for a putative methylmalonyl-CoA:CO_2 exchange reaction. Enzymatic activity was observed with an initial rate as much as 10% of the malonyl-CoA-dependent reaction. An exchange reaction using succinyl-CoA as substrate could not be detected. Furthermore, methylmalonyl-CoA functioned

Table 2: Methylmalonyl-CoA Affecting the Malonyl-CoA:$^{14}CO_2$ Exchange Activity and Acting as Substrate of a Methylmalonyl-CoA:$^{14}CO_2$ Exchange[a]

substrates	addition	product isolated	radioactivity incorporated
malonyl-CoA		malonyl-CoA	2.5 kBq
malonyl-CoA	methylmalonyl-CoA	malonyl-CoA	0.5 kBq
methylmalonyl-CoA		methylmalonyl-CoA	0.3 kBq

[a] Stilbene synthase (10 μg) and 1.0 mM malonyl-CoA were incubated with 4 nmol of $NaH^{14}CO_3$ (2.0 GBq/mmol) in 100 μL of 40 mM phosphate buffer (pH 8.0) in the presence or absence of 1.0 mM methylmalonyl-CoA.

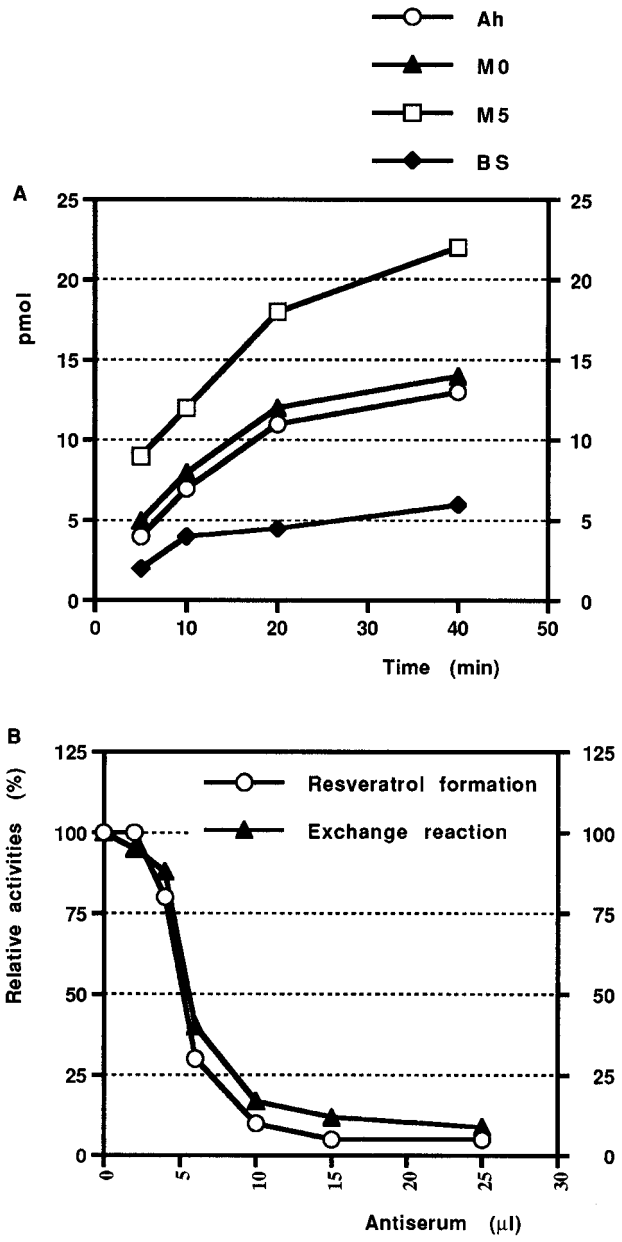

FIGURE 2: Malonyl-CoA:CO_2 exchange analyzed using various purified stilbene synthases, malonyl-CoA, and [^{14}C]bicarbonate. (A) Stilbene synthases (58 ng each) from *Arachis hypogaea* (Ah) and the recombinant grapevine enzymes M0 (M0) and M5 (M5) and a bibenzyl synthase from orchid (BS) were used for the time course. The reaction was followed by analyzing the production of labeled malonyl-CoA at various time periods. (B) Using partially purified preparation of stilbene synthase M0, we assayed both the resveratrol-forming activity and the malonyl-CoA:CO_2 exchange activity remaining in the supernatant following immunotitration with antiserum.

as a competetive inhibitor to the malonyl-CoA:CO_2 exchange reaction (Table 2).

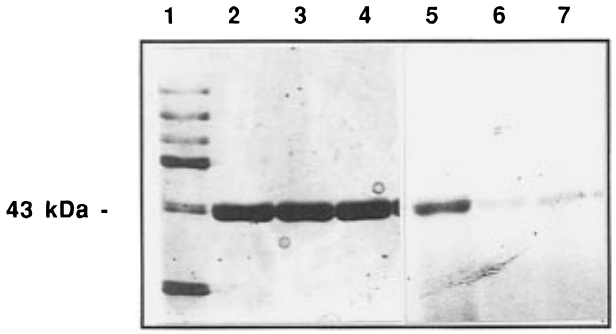

FIGURE 3: Chemical modification of stilbene synthase by reaction with [2-^{14}C]malonyl-CoA. Lanes 1−4, protein stain; lanes 5−7, fluorography. Lanes 2 and 5, product of the conversion of 25 μg of peanut stilbene synthase with 20 μM labeled malonyl-CoA; lanes 3 and 6, as lanes 2 and 5 but in the presence of 45 μM methylmalonyl-CoA; lanes 4 and 7, grapevine stilbene synthase M0 form carrying a mutation C164A (M9) incubated with [2-^{14}C]-malonyl-CoA; lane 1, marker proteins.

Stilbene synthases also possess low malonyl-CoA decarboxylase activity. With [1,3-^{14}C]malonyl-CoA as sole substrate, we followed the formation of $^{14}CO_2$. Using stilbene synthase M0 and malonyl-CoA at concentrations saturating for the chain-lengthening reaction, the decarboxylation reaction was considerably slower (5 pkat/mg of protein) than the conversion of malonyl-CoA into the stilbene resveratrol (data not shown). Using 140 μM malonyl-CoA, a rather unusual concentration in vivo, we observed a maximal velocity of 200 pkat/mg of protein in vitro. V_{max} refers to μmol of CO_2 released.

Reaction with [2-^{14}C]malonyl-CoA leads to a chemical modification of stilbene synthase. When various forms of purified stilbene synthase were incubated with [2-^{14}C]-malonyl-CoA we observed the formation of radioactively labeled protein. Applying rigorous and stringent purification methods, we demonstrated the presence of a covalently modified protein and characterized it by SDS−PAGE (Figure 3). The fluorography also shows that methylmalonyl-CoA competes with the malonyl-CoA-dependent modification reaction.

Further Characterization of the Malonyl-CoA Binding Site. The inhibition studies performed with methylmalonyl-CoA indicate that the interaction between the stilbene synthase and malonyl-CoA is specific as far as only methylmalonyl-CoA can interfere in a competitive way. It is noteworthy that methylmalonyl-CoA interfered with all reactions taking place with malonyl-CoA. The other result is, and this was corroborated by all following experiments, that in cases where malonyl-CoA conversions were affected, the conversion to resveratrol was influenced in the same way (data not shown), suggesting that the malonyl-CoA-dependent steps precede resveratrol formation.

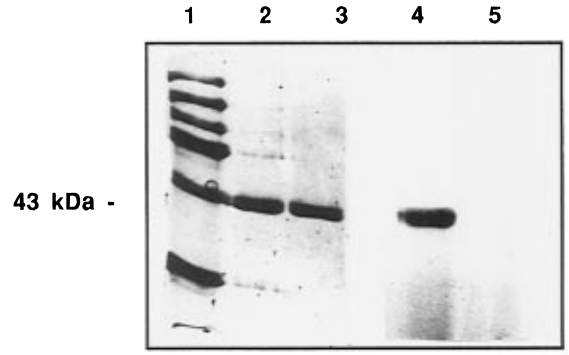

FIGURE 4: Reaction with [2-^{14}C]malonyl-CoA of stilbene synthase M0 and the M0 form reacted with *N*-ethylmaleimide. The enzymes were tested for their capacity to covalently modify themselves. After incubation of the enzymes with the radioactive malonyl-CoA, analysis of the protein was carried out by SDS−PAGE and photoimaging. Lanes 1−3, protein stain; lanes 4 and 5, fluorography. Lanes 2 and 4, M0; lanes 3 and 5, modification reaction performed after preincubation of M0 with 50 μM *N*-ethylmaleimide.

In further characterizing the malonyl-CoA binding site, we proved cysteine-164 to be the putative site of the enzyme's chemical modification by [2-^{14}C]malonyl-CoA. Earlier experiments with various stilbene synthases have shown that a thiol group is essential for the catalytic activity of resveratrol formation. Lanz *et al.* (1991) applied site-directed mutagenesis to find out which of the eight thiol groups in the peanut stilbene synthase is essential for activity. They found that exchanging cysteine residues by alanine residues in most cases greatly reduced the activity of resveratrol formation, with the mutation in C-164 having the most dramatic effect. We suspected that thiol groups may be essential for both the malonyl-CoA binding site and the chain elongation to resveratrol.

To assign the particular thiol group, we used both the stilbene synthase M0 deactivated and modified by reaction with *N*-ethylmaleimide and a M0 form carrying a mutation C164A, designated M9. We tested these forms for their capacity to covalently modify themselves by reaction with [2-^{14}C]malonyl-CoA. In both cases, the capability for this reaction taking place with the wild type enzyme was abolished (Figure 4). Furthermore, we subjected the M0 protein modified by reaction with [*ethyl*-^{3}H]-*N*-ethylmaleimide to exhaustive proteolysis with Glu-C. After separating the peptide fragments by reversed phase HPLC, we isolated one radioactively labeled fragment which was subjected to N-terminal sequencing. The sequence found (TSVRRVMLY) corresponds to the range of amino acid residues 158−167 in the recombinant grapevine stilbene synthase M0 (i.e., 151−160 in the wild type form).

From earlier investigations (Liswidowati *et al.*, 1991) it was known that stilbene synthases possess a rather exposed thiol group which may be used for purification of the enzyme by binding it to mercuri agarose and removing it using dithiothreitol. Here, we used the exposed thiol group to modify the enzyme by reacting it with ^{203}Hg-labeled *p*-hydroxymercuribenzoate. Following purification, the protein was subjected to proteolysis with Glu-C. Isolation of the labeled peptide fragment, removal of the mercuribenzoate group, and sequence determination identified a fragment which encompassed, according to its N-terminal sequence, a region identical to the fragment mentioned above. Both peptides, selected on the basis of their radioactive label, were characterized by a sequence TSVRRVMLY. This indicates that both chemical modifications brought about by reaction with *N*-ethylmaleimide or mercuribenzoate, respectively, take place at the cysteine-164 of the wild type grapevine stilbene synthase.

Table 3: Analysis of the Hydrolyzable Moiety in Different Forms of Stilbene Synthases Covalently Modified by Incubation with [2-^{14}C]Malonyl-CoA[a]

	radioactivity (Bq)		
		products of hydrolysis	
form of stilbene synthase	covalently bound to the enzyme	malonic acid	acetic acid
peanut wild type enzyme	4.8	1.1	3.7
grapevine M0 form	8.8	2.1	6.7
grapevine M5 form	11.8	2.1	9.7
pine wild type enzyme	10.1	3.3	6.8
orchid fusion protein	4.3	1.3	3.0

[a] Peanut and grapevine enzymes are resveratrol-forming stilbene synthases, the pine enzyme is a pinosylvin-forming stilbene synthase, and the orchid enzyme is a bibenzyl synthase catalyzing the malonyl-CoA-dependent conversion of *m*-hydroxyphenylpropanoyl-CoA to trihydroxybibenzyl. Following modification, the proteins were purified by gel chromatography and subjected to hydrolysis using 2 N sodium hydroxide at 25 °C for 30 min.

To gain further information about the kind of modification brought about by incubation with [2-^{14}C]malonyl-CoA we incubated the modified and then rigorously purified protein with 0.2 M hydroxylamine at 37 °C for 50 min. Subsequent gel chromatography of the protein revealed that 90% of the radioactivity formerly bound to the protein was released from it. The easy cleavage of the acyl group by neutral NH_2OH provides evidence for an acyl thioester bond.

For the characterization of the putative acyl group released we prepared various forms of modified stilbene synthases and subjected them to alkaline hydrolysis. Chemical analysis of the acid groups removed from the protein by this procedure showed that both acetyl and malonyl groups have been present on the protein (Table 3). As these acyl groups were sensitive toward reaction with hydroxylamine the combined data suggest that the chemical modification observed by reacting stilbene synthases with [2-^{14}C]malonyl-CoA afforded malonyl and acetyl thioesters.

Grapevine stilbene synthase M0 was prepared from *E. coli* cells grown in the presence of [^{32}P]-*ortho*-phosphate and peanut stilbene synthase was obtained from cell cultures grown on a [^{32}P]-*ortho*-phosphate-containing medium. Following immunoprecipitation, we were unable to detect any labeling by phosphate in the de novo synthesized enzymes indicating the absence of a phosphopantetheine structure. Likewise, the analysis of fragmentation pattern provided by electrospray ionization mass spectrometry demonstrated that stilbene synthase lacks a phosphopantetheine group. In this respect, stilbene synthases resembles chalcone synthases (Kreuzaler *et al.*, 1978).

Stilbene synthases possess seven cysteine residues, and thiol side chains other than in Cys-164 may contribute to the nucleophile formation required for Claisen condensation. Despite the lack of a thiol group on a phosphopantetheine arm, arguments can be put forward that a second sulfhydryl group is involved. We used several recombinant forms of grapevine stilbene synthase M0 (Table 4) to address this question. We proceeded by assessing the structural requirements for malonyl-CoA decarboxylation and malonyl-CoA-

Table 4: Survey of Mutant Forms Analyzed for Partial Reactions with Malonyl-CoA and the Total Reaction Leading to Resveratrol[a]

form	mutation	malonyl-CoA decarboxylase	malonyl-CoA:CO_2 exchange activity	covalent modification with malonyl-CoA	resveratrol formation
M0		100	100	100	100
M1	C60A	<5	<5	<5	<5
M2	Δ(K53,K54,K55)	10	5	<5	<5
M3	A37P	100	100	100	80
M4	S250A	60	nd[b]	20	<5
M5	N249D, A255D	65	110	80	50
M6	C130A	60	nd	50	50
M7	C347A	100	100	nd	80
M8	Δ(264−392)	30[c]	nd	?	<1
M9	C164A	<5	nd	<5	<5
M10	VSI-(230−233)EAAD	110	nd	100	75

[a] The conversion rates are related to M0 and are given in %. [b] nd, not determined. [c] Activity decreases rapidly during purification.

dependent covalent enzyme modification with respect to the possible role of further cysteine residues; e.g., on the basis of sequence homology with the active centers of 3-hydroxy-3-methylglutaryl-CoA synthase we chose the thiol group of Cys-60 as a potential nucleophilic agent acting in malonyl-CoA-dependent reaction.

Mutations C60A and Δ(K53,K54,K55) led to the proteins designated M1 and M2, respectively. Both mutant forms were characterized by their virtual lack of any malonyl-CoA-dependent partial activity (Table 4). This indicates that Cys-60 and its environment have to be implicated in the transfer of malonyl-CoA preceding the resveratrol formation. A change A37P at a putative turn in the secondary structure did not alter any of the partial activities, neither the malonyl-CoA decarboxylation nor the resveratrol formation. Another mutation in the N-terminal half of the enzyme sequence, M6 (C130A), was used to test a potential role for Cys-130. Unlike M1, the activities of M6 were only slightly reduced.

Characterization of a Putative p-Coumaroyl-CoA Binding Site by Affinity Labeling. For all stilbene synthases tested, *p*-azidocinnamoyl-CoA (K_i = 9.4 μM) was found to be an efficient inhibitor competing with *p*-coumaroyl-CoA. However, *p*-azidocinnamoyl-CoA was not converted into a stilbene derivative. Furthermore, *p*-azidocinnamoyl-CoA was bound to the enzyme and subsequently covalently linked to neighboring amino acid residues via photochemical nitrene formation. The outcome was that the resveratrol formation was reduced, the chain lengthening being much more affected than the preceding malonyl-CoA:CO_2 exchange (Figure 5).

In addition, we prepared 50 μg of photolabeled enzyme and subjected it to an extensive but not complete proteolysis by Glu-C. Following separation and purification of the cleavage products, we selected two fragments on the basis of their intensive absorption of the cinnamoyl residue at 262 nm (see Experimental Procedures) and determined the N-terminal sequence. This led to the characterization of a peptide I with the N-terminal sequence DALDSLVG and a peptide II with the N-terminal sequence RPLFQLVS. The N-terminus of peptide I corresponds to amino acid residues 204−211 of the wild type grapevine stilbene synthase (characterized in pSV25), and the N-terminal region of peptide II is identical to amino acid residues 234−241. Thus, the putative binding site for malonyl-CoA, which is in the N-terminal region of the stilbene synthase and requires at least the stretch between residues 50−170, is separated from the *p*-coumaroyl-CoA binding site requiring the middle and the C-terminal parts of the enzyme molecule.

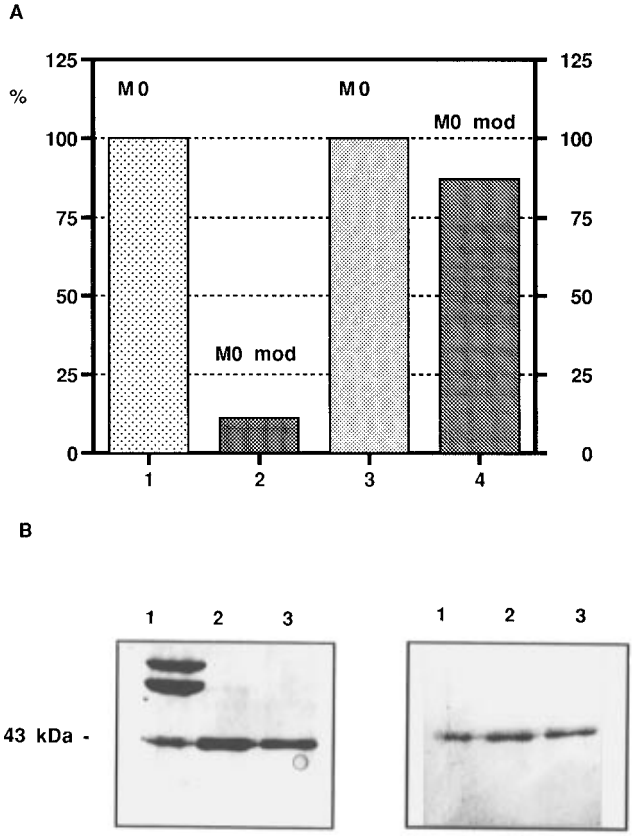

FIGURE 5: Differential effect of *p*-azidocinnamoyl-CoA on the binding sites for *p*-coumaroyl-CoA and malonyl-CoA, respectively. (A) Effect of the photoaffinity labeling with *p*-azidocinnamoyl-CoA (lanes 2 and 4) on the chain-lengthening reaction (lanes 1 and 2) and the malonyl-CoA:CO_2 exchange (lanes 3 and 4). (B) Enzyme labeling by reaction with [2-^{14}C]malonyl-CoA; *p*-azidocinnamoyl-CoA is not interfering with the malonyl-CoA binding site as shown by the unreduced formation of chemically modified enzyme in the presence of *p*-azidocinnamoyl-CoA. Protein stain (left) and fluorography (right). Lane 1, 7.5 μg of stilbene synthase M0, 15 μg of bovine serum albumin, and 15 μg of human transferrin; lane 2, M0; lane 3, M0 incubated with 0.1 mM *p*-azidocinnamoyl-CoA.

We examined the binding of *p*-azidocinnamoyl-CoA also by a second means which is able to differentiate between the *p*-coumaroyl-CoA binding and malonyl-CoA binding sites, i.e., the covalent chemical modification of the enzyme by acting on [2-^{14}C]malonyl-CoA. As seen in Figure 5B, this modification reaction was not prevented by the presence of *p*-azidocinnamoyl-CoA.

On the basis of the preferable binding of *p*-azidocinnamoyl-CoA to amino acid residues lying in the middle or

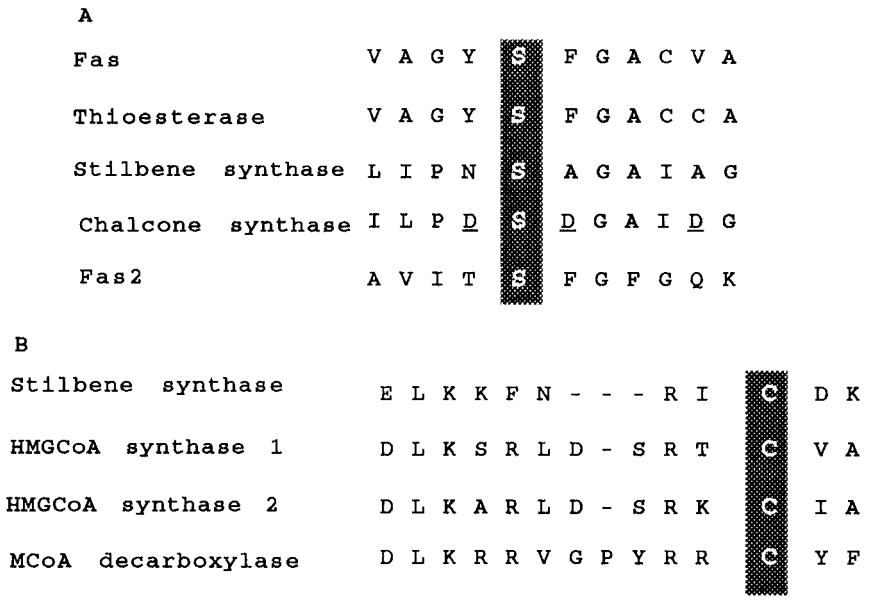

FIGURE 6: Comparison of stilbene synthase sequence encompassing potential active sites. Ser-250 and its environment are compared with the potential active centers of enzymes possessing thioesterase activity (A). Rat fatty acid synthase (Fas; Amy *et al.*, 1989) and a bacterial thioesterase (Li *et al.*, 1996) were included for comparison. The respective chalcone synthase sequence clearly differs by the three charged residues (D). The respective sequence ITSFG of yeast Fas2 (Mohamed *et al.*, 1988) indicates that deviations from the consensus sequence GxSxG are allowed. Part B shows Cys-60 of stilbene synthase aligned with sequences of malonyl-CoA decarboxylase from uropygial gland (MCoA decarboxylase; Jang *et al.*, 1989) and with two forms of a hydroxymethylglutaryl-CoA synthase (HMGCoA synthase; Kattar-Cooley *et al.*, 1990).

the C-terminal part of the stilbene synthase molecule, but certainly not to the N-terminal region, and *p*-azidocinnamoyl-CoA not interfering with the binding and modification at the malonyl-CoA binding site, it would be tempting to conclude simply that the two binding sites, although forced to interact during chain lengthening, are separated from each other. This is corroborated by the findings that mutant M8 constructed as the deletion of C-terminal amino acid residues ($\Delta(264-392)$) lacked any stilbene-forming activity but possessed a significant amount of malonyl-CoA decarboxylase activity.

After malonyl-CoA and *p*-coumaroyl-CoA are bound to the synthase, a condensation reaction should proceed which may have similarities to the condensation reaction of fatty acid synthases and 6-methylsalicylic acid synthase (Omura, 1976). In those cases, the antibiotic cerulenin [(2*S*,3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide] from *Cephalosporium caerulens* was used as inhibitor to characterize the thiol group involved in the condensation reaction (Omura, 1976; Kauppinen *et al.*, 1988; Morisaki *et al.*, 1993). Using stilbene synthase M0 and 0.1 mM cerulenin, we observed an 80% inhibition in the resveratrol formation. The level of inactivation of stilbene synthase M0 and M5 (0.5 μM) by cerulenin (100 μM) was also determined after the enzyme had been preincubated with *p*-coumaroyl-CoA. Preincubation with *p*-coumaroyl-CoA (300 μM) was found to protect the enzyme from inactivation, whereas preincubation with malonyl-CoA (300 μM) had virtually no protective effect.

During the sequence of reactions postulated for the formation of hydroxystilbenes like resveratrol, an intermediary structure of a stilbenecarboxylic acid CoA-ester may be proposed. This intermediate should eventually be converted to the stilbene by a thioesterase and decarboxylase (see Figure 1). Therefore, the primary sequence of stilbene synthases was screened for putative thioesterase motifs. To obtain data hinting at the presence of such an activity we used diisopropyl fluorophosphate as inhibitor. We observed a significant inhibition when the enzyme was incubated with diisopropyl fluorophosphate 30 min at 30 °C prior to the addition of the substrates. Applying 5 mM diisopropyl fluorophosphate in this way resulted in 90% inhibition. The effect could not be reversed by preincubation with diisopropyl fluorophosphate in the presence of *p*-coumaroyl-CoA.

In line with this experiment, we looked for a potential candidate of the thioesterase active center. As the reaction sequence catalyzed by chalcone synthase does not include an esterase and decarboxylase step, we also concentrated on differences between the structures of stilbene synthases and chalcone synthases. On the basis of sequence comparisons with thioesterase consensus sequences (Figure 6), Ser-250 and its environment were thus assumed as candidates for an active center. Mutation S250A (in protein M4) did not alter the malonyl-CoA decarboxylase or the malonyl-CoA:CO_2 exchange reaction but greatly reduced the stilbene formation (Table 4). Mutation in the environment of Ser-250 did not cause significant reduction of activities, as shown with M5 (N249D,A255D). As expected, M5 was susceptible to chemical modification by [2-^{14}C]malonyl-CoA.

Studies To Assign a Putative m-Hydroxyphenylpropionyl-CoA Binding Site. The orchid bibenzyl synthase has previously been characterized by its clear preference of *m*-hydroxyphenylpropionyl-CoA as substrate instead of the *p*-hydroxyphenylpropenoyl-CoA (*p*-coumaroyl-CoA) used by the resveratrol-forming stilbene synthases. The two CoA esters functioning as starters in the chain elongation process differ in two respects: (a) α,β-unsaturated versus saturated fatty acid moiety and (b) *para* versus *meta* substitution pattern. By sequence comparison and looking for changes between stilbene synthases and bibenzyl synthase we selected the stretch DEAADE in bibenzyl synthase (residues 229−234 in wild type enzyme) compared to DVSI-E in stilbene synthase (residues 229−234 in wild type enzyme) as the

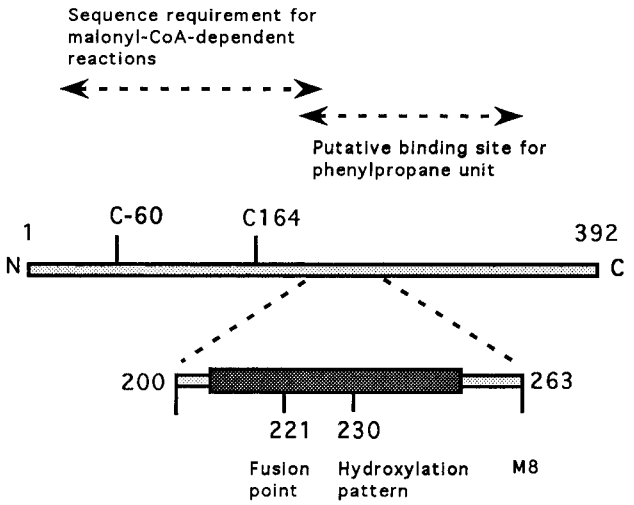

FIGURE 7: Map of the stilbene synthase molecule of the sites addressed in the present study. The amino acid residues are numbered as they occur in the wild type grapevine stilbene synthase. As phenylpropane units, we tested *p*-hydroxyphenylpropenoyl-CoA (*p*-coumaroyl-CoA), *m*-hydroxyphenoylpropanoyl-CoA, and *p*-azidophenylpropenoyl-CoA. The fusion point indicates the site where stilbene synthase and bibenzyl synthase were fused alternately to obtain mutants M12 and M13. The mutation site where marked differences in the preferrence of *m*- versus *p*-hydroxylated substrates was observed is labeled "hydroxylation pattern" while the region covalently modified with *p*-azidophenylpropenoyl-CoA is indicated as a black bar. With M8, we indicate the length of the deletion mutant still showing malonyl-CoA decarboxylase but no capacity of resveratrol formation.

putative site crucial for binding of *meta*- or *para*-hydroxylated substrates. The mutant protein M10 deriving from stilbene synthase M0 but carrying EAAD instead of VSI- was characterized by full activities in all malonyl-CoA-dependent partial reactions but showed a slightly reduced resveratrol-forming activity (Table 4).

With their respective substrates, homogenous stilbene synthase exhibits a specific activity 5 times as high as that of purified bibenzyl synthase. With M0, the conversion rate of *m*-hydroxyphenylpropionyl-CoA to the bibenzyl was less than 0.35% of the conversion of *p*-coumaroyl-CoA to resveratrol. In M10, the conversion rate of *p*-coumaroyl-CoA to resveratrol was 75% of the rate observed with M0 while the ratio of conversion of *m*-hydroxyphenylpropionyl-CoA relative to *p*-coumaroyl-CoA increased to 0.11 upon this mutation. This indicates that the preference for *meta*-hydroxylated substrates was indeed attained by the exchange of the four amino acids. The findings also show that the site for selecting the phenylpropane unit is within the C-terminal half of the enzyme molecule.

With respect to the preference of *meta*- and *para*-hydroxylated substrate, we fused an N-terminal part of the stilbene synthase M0 with an C-terminal part of a bibenzyl synthase (M12) as well as an N-terminal part of the bibenzyl synthase with the C-terminal part of stilbene synthase (M13). Choosing a common restriction enzyme site for stilbene synthase M0 and bibenzyl synthase cDNA we made constructs by cleaving the cDNAs at nucleotide 701 (of SV25) and fusing the two fragments obtained in a reciprocal manner (see Figure 7). Thus, the respective proteins were composed of part I of stilbene synthase and part II of bibenzyl synthase (M12, Table 1) and vice versa (M13, Table 1). While the absolute activities of these recombinant proteins decreased compared to the wild type form, it was still possible to test them with *p*-coumaroyl-CoA and *m*-hydroxyphenylpropionyl-CoA as substrates. Applying the ratio x for the conversion rates of *p*-hydroxylated educt/*m*-hydroxylated educt we found a preference ($x = 4.04$) for the *p*-hydroxylated compound when the N-terminal region originated from the stilbene synthase, and a preference for *meta*-hydroxylated educt ($x = 0.38$) when the N-terminal region was from the bibenzyl synthase. Thus, the selectivity for the aromatic substrate should reside within the region upstream of the fusion point used here, i.e., amino acid residue 221.

DISCUSSION

Many aspects of the biosynthesis of cyclic polyketides resemble those of fatty acid synthesis in terms of both chemistry and enzymatic mechanism, despite the great variety in the polyketide structures that are formed. For instance, both syntheses employ repeated Claisen condensations for acyl chain elongation, usually involving acetyl-CoA and malonyl-CoA and, to a lesser extent, butyryl CoA, propionyl-CoA, and methylmalonyl-CoA. For the first time with polyketide synthases, we provide further details concerning the binding and activating of malonyl-CoA as chain-lengthening reagent.

The mode of Claisen condensation between an aromatic CoA ester and the carbanion-providing malonyl-CoA implicates several partial reactions (Figure 1). The reactions with malonyl-CoA take place in the absence of the second substrate, and we do not know whether these steps are prerequisites of the Claisen condensation or side reactions. However, the present data obtained with stilbene synthases suggest that all conversions observed with malonyl-CoA proceed at the same center which also is involved in the consecutive chain lengthening because the inhibitory effects and mutations all influenced the various processes taking place with malonyl-CoA in the same way. Several mutations and modifications were found which lack the capacity of resveratrol formation but exhibited full or reduced conversion rates with malonyl-CoA while no cases were detectable vice versa. This is emphasized by the analysis of the deletion mutant form M8 lacking a large C-terminal region which has been shown to be required for binding of *p*-coumaroyl-CoA. The results agree with the concept that malonyl-CoA binding (a) precedes the condensation reaction and (b) proceeds to a large extent independently from resveratrol formation.

All stilbene synthases and bibenzyl synthases were characterized by a malonyl-CoA:CO_2 exchange reaction. Thus, the stilbene synthases tested behaved similar to the chalcone synthase from *Petroselinum hortense* (Kreuzaler *et al.*, 1978) in that they form an enzyme-bound species of acetyl-CoA. However, stilbene synthases differ from chalcone synthase as we did not find detectable amounts of side products as benzalacetones or dihydropyrones reported for chalcone synthase (Hrazdina *et al.*, 1976). Likewise, as to the sequential order of reactions, the kind of ring closure catalyzed by stilbene synthase deviates from the chalcone synthase steps and instead resembles the formation of 6-methylsalicylic acid (Dimroth *et al.*, 1976): the new aromatic ring includes one C-atom originating from the starter acyl group, and the product is a carboxylic acid. A carboxylic acid has been postulated for the stilbene formation

(Figure 1), and its occurrence has precedents since stilbene-2-carboxylic acids have been found in plants (Kindl, 1985). Malonyl-CoA:CO_2 exchange reaction has also been observed with β-ketoacyl-ACP synthase from *E. coli* (Alberts *et al.*, 1972). Yeast fatty acid synthase revealed a malonyl-CoA decarboxylase activity when it was treated with iodoacetamide (Kresze *et al.*, 1977).

The molecular structures of 6-methylsalicylate synthase and most other β-ketoacyl synthases (Kauppinen *et al.*, 1988) have in common a cysteine as substrate-binding site and the -SH group of a phosphopantetheine arm as the site for the second substrate. In contrast to these invariant structures in bacterial and fungal polyketide synthases, both chalcone synthase and stilbene synthase lack a phosphopantetheine arm. Thus, they are assumed to use two different cysteinyl residues for the transfer reactions. In stilbene synthases, likely candidates for this task are Cys-164 and Cys-60 (Figures 6 and 7). Originally, Cys-341 was implicated in the catalytic mechanism (Schröder & Schröder, 1990) on the basis of sequence similarities to the cerulenin-binding sites found in *E. coli* β-ketoacyl-ACP synthase (Kauppinen *et al.*, 1988). A C164A mutation has been found to abolish the capacity for resveratrol formation (Lanz *et al.*, 1991). Here we demonstrate that the transfer and reactions with the malonyl group take place at Cys-164. Chemical modification of Cys-164 by reaction with *N*-ethylmaleimide or mercuribenzoate abolished the reactions with malonyl-CoA. Likewise, mutations at Cys-60 strongly impaired the malonyl-CoA-dependent reactions.

Under physiological conditions in plants, the pathways from phenylalanine via *p*-coumaroyl-CoA to trihydroxystilbene and via *m*-hydroxyphenylpropionyl-CoA to trihydroxybibenzyl and dihydrophenanthrenes differ in many aspects (Kindl, 1985). Despite their occurrence in distinct plant families, bibenzyl synthase and stilbene synthase show a high degree of structural similarities. This led us to prepare fusion proteins which differ from the wild type forms only in terms of the overall structure. Surprisingly, the recombinant proteins (M12 and M13) had only 1/50 of the activity compared to other mutant proteins (e.g., M10 and M5) which had changes solely in the range of amino acid residues 200−270 (see Figure 7). But the crucial feature in M12 and M13 was the fact that the two partial sites fused together at Ser-221 seem to be not fully compatible and obviously do not yield an appropriate binding site for the aromatic thioester. Thus, exchanges of various small groups at particular sites in the range of amino acid residues 220−270 seem to be less harmful to the gain of stilbene-forming activity than changes in the overall topology of a broad area within the C-terminal part of the enzyme molecule.

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