Plant Polyketide Synthases: A Chalcone Synthase-Type Enzyme Which Performs a Condensation Reaction with Methylmalonyl-CoA in the Biosynthesis of C-Methylated Chalcones[†,‡]

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ABSTRACT: Heterologous screening of a cDNA library from *Pinus strobus* seedlings identified clones for two chalcone synthase (CHS) related proteins (PStrCHS1 and PStrCHS2, 87.6% identity). Heterologous expression in *Escherichia coli* showed that PStrCHS1 performed the typical CHS reaction, that it used starter CoA-esters from the phenylpropanoid pathway, and that it performed three condensation reactions with malonyl-CoA, followed by the ring closure to the chalcone. PstrCHS2 was completely inactive with these starters and also with linear CoA-esters. Activity was detected only with a diketide derivative (*N*-acetylcysteamine thioester of 3-oxo-5-phenylpent-4-enoic acid) that corresponded to the CHS reaction intermediate postulated after the first condensation reaction. PstrCHS2 performed only one condensation, with 6-styryl-4-hydroxy-2-pyrone derivatives as release products. The enzyme preferred methylmalonyl-CoA against malonyl-CoA, if only methylmalonyl-CoA was available. These properties and a comparison with the CHS from *Pinus sylvestris* suggested for PstrCHS2 a special function in the biosynthesis of secondary products. In contrast to *P. sylvestris*, *P. strobus* contains C-methylated chalcone derivatives, and the methyl group is at the position predicted from a chain extension with methylmalonyl-CoA in the second condensation of the biosynthetic reaction sequence. We propose that PstrCHS2 specifically contributes the condensing reaction with methylmalonyl-CoA to yield a methylated triketide intermediate. We discuss a model that the biosynthesis of C-methylated chalcones represents the simplest example of a modular polyketide synthase.

Chalcone synthase (CHS)[1] (EC 2.3.1.74) is a plant-specific polyketide synthase which synthesizes the precursor for a large number of biologically important secondary plant products (*1*). The enzyme uses a CoA-ester from the phenylpropanoid pathway as starter substrate (typically 4-coumaroyl-CoA), performs three condensation reactions with malonyl-CoA, and folds a tetraketide intermediate to a new aromatic ring system. CHS is a member of a protein family that includes the closely related acridone and stilbene synthases which are present in a relatively small number of plants. These enzymes also perform three condensation reactions, but they are distinguished from CHS by other substrate specificities or by the type of aromatic ring that is formed in the reaction. The proteins are more than 65% identical among each other, and they have no significant sequence similarity to other polyketide synthases (reviewed in ref (*2*).

Of the more than 100 entries labeled as CHS in the public databases from sequence similarity analysis, only 20% have been confirmed from functional evidence, and the assignment of the other sequences must be considered as preliminary. One reason is that it is not possible to distinguish CHS, stilbene synthase, or acridone synthase at the sequence level, and in at least one case, it is known that a CHS-related protein does not have any of these functions (*3*). Another reason is the emerging evidence from enzyme activity studies that the protein family probably contains additional members which are functionally distinguished by still other starter substrate specificities (*4, 5*) or by the use of only one or two condensation reactions (*6, 7*).

We describe a new and unexpected function for a CHS-type enzyme. The results with a protein cloned from *Pinus*

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[‡] The nucleotide sequence data have been submitted to the GenBank/EMBL Data Bank and are available under the accession numbers AJ002156 (PstrCHS2) and AJ004800 (PstrCHS1).

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[1] Abbreviations: CHS, chalcone synthase; methylstyrylpyrone, 3-methyl-6-styryl-4-hydroxy-2-pyrone; NAC, *N*-acetylcysteamine-thioester; NAC-diketide, *N*-acetylcysteamine thioester of 3-oxo-5-phenylpent-4-enoic acid; PstrCHS1, CHS1 from *P. strobus*; PstrCHS2, CHS2 from *P. strobus*; PsylCHS, CHS from *P. sylvestris*; pyrone, 4-hydroxy-2-pyrone; styrylpyrone, 6-styryl-4-hydroxy-2-pyrone; TLC, thin-layer chromatography.

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strobus L. (Eastern white pine) suggest that it is an enzyme performing with methylmalonyl-CoA as chain extender a condensation reaction in the biosynthesis of C-methylated chalcones.

EXPERIMENTAL PROCEDURES

Materials. [2-^{14}C]Malonyl-CoA (55 mCi/mmol) and D,L-2-[methyl-^{14}C]methylmalonyl-CoA (56.4 mCi/mmol) were obtained from Biotrend (Cologne, Germany). Other CoA-esters were from the laboratory collection or purchased from Sigma Biochemicals (München, Germany). Authentic samples of stropopinin (6-methyl-naringenin) and 6,8-dimethyl-naringenin were a gift from E. Wollenweber (Technical University of Darmstadt).

N-Acetylcysteamine-Thioesters (*NAC*). The NAC-derivative of cinnamic acid [(*E*)-phenylprop-2-enethioic acid *S*-[2-(acetylamino)ethyl]ester] (*8*) and the NAC-derivative corresponding to the product of the first condensation reaction with cinnamoyl-CoA [(*E*)-3-oxo-5-phenylpent-4-enethioic acid *S*-[2-(acetylamino)ethyl]ester] (NAC-diketide) (*9*) were synthesized via described methods. The detailed characterization of the substances is available on request.

Plant Material and Induction. Seeds from *Pinus strobus* L. (Fa. Geigle, Nagold, Germany) were germinated and grown in white light (80 W/m^2; 16 h light, 8 h dark) in a phytotron at 25 °C and with 50−60% humidity. The plantlets were used three weeks after germination, and they were induced by wounding and infiltration with a yeast extract solution as described previously (*10*).

cDNA Synthesis, Screening, and Sequence Analysis. The cDNA libraries were constructed with 5 μg of poly(A)-rich RNA from *P. strobus* and cDNA synthesis kits from Amersham Corporation (cDNA Synthesis System Plus, no. RPN1256Y) and from Pharmacia LKB Biotechnology Inc. (You-Prime cDNA Synthesis Kit no. 27-9273-01). After addition of *Eco*RI/*Not*I adaptors (5′-AATTCGCGGCCGC-3′, 5′-pGCGGCCGCG-3′, Pharmacia Biotech), the cDNAs were ligated to *Eco*RI digested phage λ gt11 (Pharmacia Biotech) (*11*) and packaged with a kit from Amersham Corporation (λ in vitro Packaging Kit no. N334L). The library was screened with the radioactively labeled CHS cDNA from Scots pine (*P. sylvestris*) (*10*) as described previously (*12, 13*). The cDNAs were sequenced by the dideoxy nucleotide chain termination technique (*14, 15*). The vectors, phages, and methods have been described (*13*). The pTZ18R and pTZ19R system, helper phage M13K07, *E. coli* strain JM109 (Pharmacia LKB Biotechnology), and the reverse sequencing primer (Boehringer Mannheim) or custom-synthesized oligonucleotides were used with subcloned cDNA fragments. DNA polymerization reactions were performed with [^{35}S]dAdo*P*[S]*PP* (adenosine 5′[1-thio]-triphosphate) (37 TBq/mmol, Amersham Corporation) and modified T7 DNA polymerase (Sequenase, United States Biochemical Corporation).

Expression in E. coli. Site-directed mutagenesis with appropriate custom-synthesized oligonucleotides was used to introduce an *Nco*I-site into the protein start codons of the cDNAs, as described previously (*16*). The mutagenesis was performed with the cDNAs cloned in vector pTZ19R (*17*), and single-stranded DNA was obtained with helper phage M13K07 in *E. coli* strain RZ1032 (*18*). The mutations were verified by DNA sequence analysis. The cDNAs were then cloned with *Nco*I/*Hin*dIII into expression vector pQE−6 (*19*) (*P. strobus* CHS1, PstrCHS1), or with *Nco*I/*Not*I into pQE 6 modified by the introduction of a *Not*I site (*16*) (*P. strobus* CHS2, PstrCHS2). This joined the protein-coding regions via the *Nco*I site directly with the optimal promoter-translation-start configuration of the vector. The plasmid overexpressing the CHS from *Pinus sylvestris* L. (PsylCHS) was obtained by recloning the coding region from vector pKK233-2 (*10*) into pQE-6 (*19*). The proteins were expressed in *E. coli* strain RM82 that contained an additional plasmid (pUBS520) with the *argU* (*dnaY*) gene that improves the expression of eucaryotic proteins (*20*) (a gift from R. Mattes). The induction for protein expression has been described (*21*).

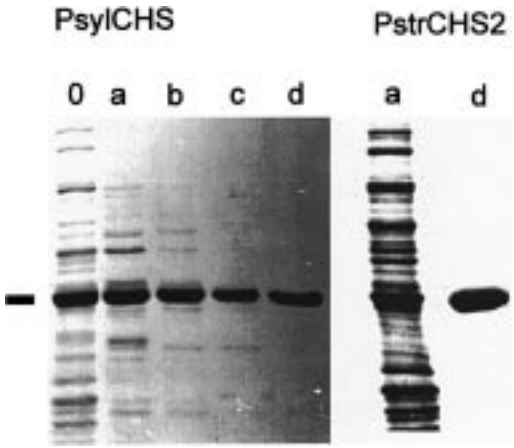

FIGURE 1: Protein purification. The proteins in the fractions were separated by SDS gelelectrophoresis and stained with Coomassie-Serva Blue R250. PsylCHS: 0, crude extract 50 μg, (a) ammonium sulfate precipitate (45 μg); (b) hydrophobic interaction chromatography (10 μg); (c) size exclusion chromatography (10 μg); (d) anion exchange chromatography (5 μg). PstrCHS2: (a) ammonium sulfate precipitate (56 μg); (d) anion-exchange chromatography (7 μg). The steps are described in detail in the Experimental Procedures. The bar at the left indicates the size of the subunits (43 kDa) of PsylCHS and PstrCHS2.

Enzyme Purification. With both PstrCHS2 and PsylCHS, the purification could be followed by visual inspection of the fractions stained with Coomassie-Serva Blue R250 after SDS gel electrophoretic separation because the proteins constituted 2−4% (PstrCHS2) or 5−10% (PsylCHS) of the total soluble protein in crude extracts. The harvested cells were resuspended in 0.1 M Tris-HCl (pH 8) containing 1 mM dithiothreitol, broken in a French pressure cell (1018 psi), and centrifuged to remove insoluble material (20 min 20000*g* at 4 °C).

The purifications used four steps in various combinations: (a) fractionated ammonium sulfate precipitation, (b) hydrophobic interaction chromatography (Fractogel EMD Butyl 650 S, Merck, Germany), (c) size exclusion chromatography (Fractogel EMD BioSEC S, Merck, Germany), and (d) anion-exchange chromatography (Fractogel EMD DEAE 650 S, Merck, Germany). The procedures followed the recommendations supplied by the manufacturer. All buffers contained 1 or 2 mM dithiothreitol. PstrCHS2 was purified through step a, with the fraction from 40 to 65% saturation containing most of the protein, and step d, in which it eluted at 0.14 M NaCl as a distinct peak and separate from the majority of other proteins. Stained gels (Figure 1) showed

Table 1: Purification of the CHS from *P. Sylvestris* Expressed in *E. coli*

purification step	protein (mg)	specific activity (nmol/s/mg)	purification (-fold)
crude extract	810	10.2	1
(a) ammonium sulfate (60−80%)	108	19.0	1.9
(b) fractogel EMD Butyl 650 S	42	23.3	2.3
(c) fractogel EMD BioSEC S	21	32.0	3.1
(d) fractogel EMD DEAE 650 S	13	33.6	3.3

that the protein was about 90−95% pure, with a few proteins of smaller size (not visible in Figure 1). They reacted with antiserum against CHS, indicating that they represented degradation products of the enzyme. PsylCHS was purified through all four steps to apparent homogeneity (Figure 1). In step a, most of the protein was in the fraction from 60 to 80% saturation; it eluted in step b at 0.7−0.8 M ammonium sulfate (gradient from 1.5 to 0 M ammonium sulfate), in step c) at the size of dimers (70−90 kDa), and in step d at 80 mM NaCl (step gradient from 0 to 1 M NaCl). The purification is summarized in Table 1.

Enzyme Assays and Evaluation. The preparation of crude extracts has been described (*21*). Standard incubations (0.1 mL) contained 10 μM starter CoA-ester, 16 μM [^{14}C]-malonyl-CoA (73 000 dpm) or 8.9 μM [^{14}C]methylmalonyl-CoA (110 000 dpm), 0.1 M HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]) adjusted to pH 7, and 3−10 μg protein. They were stopped after 20 or 30 min at 37 °C by addition of 25 μL of 1 M Tris-HCl (pH 9.0) to convert the chalcones into the isomeric flavanones. After a further 10 min at 37 °C, the products were extracted into ethyl acetate and subjected to quantitative thin-layer-chromatographic analysis (TLC) (*22*) with 20% aqueous acetic acid as solvent. The amount of enzyme and the length of incubation were chosen not to exceed the incorporation of 25% of the radioactive substrate.

Most of the experiments with purified enzymes were performed with the assay conditions optimized for reactions with methylmalonyl-CoA. The modifications were a volume of 50 μL, the use of 20 μM starter CoA-ester, and 17.7 μM [^{14}C]methylmalonyl-CoA (110 000 dpm). The incubations were at pH 6 and at 30 °C, and the products were extracted at pH 6 or pH 5. Other variations, in particular for the chain extender competition experiments, are described in the text or in the figure legends. The incubations optimized for identification of the products contained 0.1 M HEPES adjusted to pH 6, 5−15 μg of protein, 20 μM starter CoA-ester, and 15 μM unlabeled chain extender (methylmalonyl-CoA or malonyl-CoA) in a final volume of 0.1 mL.

LC/MS. The LC/negative ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 3.5 kV; APICID offset voltage 10 eV; heated capillary temperature 220 °C; sheath gas nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 μm, 1 × 100 mm, SEPSERV). The HPLC conditions were the following: gradient system starting from 20% CH_3CN in H_2O (each of them contained 0.2% acetic acid) to 90% CH_3CN in H_2O within 10 min; flow rate 70 μL/min; detection at 284 nm. All mass spectra were averaged and background subtracted.

Data for the 3-methyl-6-styryl-4-hydroxy-2-pyrone synthesized in vitro (in brackets, *m*/*z* relative intensity): R_t 10.97 min, $[M - H]^-$ 227 (40), $[M - H\text{-}CO_2]^-$ 183 (100). The results were in agreement with those of an authentic sample.

GC-EIMS of Trimethylsilyl Ethers (*TMSi*). The GC-EIMS measurements were carried out with a MD 800 (Fisons Instruments) using the following conditions: EI (70 eV), capillary column DB-5MS (15 m × 0.32 mm, 0.25 μm film thickness), source temperature 200 °C, injection temperature 250 °C, interface temperature 300 °C, carrier gas He, flow rate 1.3 mL/min; and splitless injection; temperature program, 3 min at 80 °C, then elevated to 290 °C with 10 °C/min, then hold at 290 °C for 10 min. The relative retention times (RR_t) were calculated with respect to squalene (R_t = 18.94 min).

Data for the substances synthesized in vitro (in brackets, *m*/*z* relative intensity), 3-methyl-6-styryl-4-hydroxy-2-pyrone: RR_t 0.87, 300 (M^+, 100), 285 ($[M - Me]^+$, 14), 272 ($[M - CO]^+$, 43), 257 ($[M - Me\text{-}CO]^+$, 21), 241 ($[M - Me\text{-}CO_2]^+$, 19), 182 (12), 131 ([C6H5−CH═CO]$^+$, 24), 103 ([C6H5-CH═CH]$^+$, 45), 73 ($TMSi^+$, 91). The data were in agreement with those of an authentic sample. 3,6-Dimethyl-4-hydroxy-2-pyrone: RR_t 0.36, key ions of the trimethylsilyl ether at *m*/*z* 212 (M^+, 16), 197 ($[M - Me]^+$, 7), 184 ($[M - CO]^+$, 57), 169 ($[M - CO\text{-}Me]^+$, 25), 153 ($[M - Me\text{-}CO_2]^+$, 4), 141 ($[M - Me\text{-}2CO]^+$, 11), 73 ($TMSi^+$, 100), 43 ($CH_3\text{-}CO^+$, 38). The properties have been described previously (*23*).

Other Techniques. Standard procedures of molecular biology were performed with established techniques (*12*).

RESULTS AND DISCUSSION

Only One of Two Closely Related CHS-Type Proteins Cloned from P. strobus has CHS Activity. The screening of a *P. strobus* cDNA library with the CHS from *P. sylvestris* identified two CHS-related sequences (PstrCHS1 and PstrCHS2), and both were obtained as full-length clones with respect to the protein-coding regions. The deduced proteins (Figure 2) were 87.6% identical. Both were expressed in *E. coli*, and immunoblots showed that the proteins were present in similar amounts. Assays with PstrCHS1 in crude extracts revealed the activities typical for CHS-type proteins in the biosynthesis of chalcones, with substrate preferences very similar to those of the CHS from *P. sylvestris* L. (Table 2). In contrast to PstrCHS1, PstrCHS2 was inactive with any of these substrates.

The inactivity of the protein suggested the possibility that the cDNA contained, introduced as artifacts during the cloning steps, sequence changes that inactivated the protein without interrupting the open reading frame. This seemed unlikely because PstrCHS2 was obtained in two independent cDNAs that only differed in their lengths at the 5′- and 3′-ends, but otherwise were identical. However, a look at sequence motifs highly conserved in CHS revealed a potentially significant difference. The sequences surrounding the active site of the condensing reaction are highly conserved (*22*). PstrCHS1 and all other CHSs proven to be functional contain Phe or rarely Tyr directly after the Cys of the active site (position 169), but PstrCHS2 contained Val (Figure 2). Using unique restriction sites common to both cDNAs, we constructed protein hybrids between PstrCHS1 and PstrCHS2 to test the importance of this exchange. The constructs and the results are summarized in Figure 3. The

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a) PstrCHS1  MPGGMMADLEAFRKAQRADGPATILAIGTATPPNAVDQSTYP
b) PstrCHS2  ...TLGMNF..I.................N........S..
c) PsylCHS   .AA...K..............................S..

               SacI
                ↓56
a) DYYFKITNSEHMTELKEKFRRMCDKSGIKKRYMYLTEEILNENPSVCAYMAP
b) .F..R.............Q........N...........KA..NL.D.K.T
c) ........................A.............K...K..E....

a) SLDARQDMVVVEVPRLGKEAAAKAIKEWGQPKSKITHVIFCTTSGVDMPGAD
b) ..............K.....................................
c) .....................................................

                                    BglII
                          169↓↓170      ↓181
a) YQMTKLLGLRPSVKRVMMYQQGCFAGGTVLRVAKDLAENNRGARVLVVCSEI
b) .HL.R....SL............V.......L.....................
c) ..L.....................................................

a) TAVTFRGPSDTHLDSMVGQALFGDGARALIVGADPVPEVEKPCFEMLWTAQT
b) .........G.......D........S.I......I.........L......
c) ........................A..................L......

a) ILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLVEAFQQFGISDWNQ
b) .I.Y.........N.S...........A...N..G.........Y......K
c) .....................................................

a) LFWIAHPGGPAILDQVEAKLNLDPKKLRATRQVLSEYGNMSSACVHFILDEM
b) ..................T....K.E..........................
c) ............................S.......D............L...

a) RKSSQQNGCSTTGEGLDVGVLFGFGPGLTVETVVLKSVPLQ      395
b) ....R.........................I...........      395
c) ....KEK................................LD     396
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FIGURE 2: Comparison of PstrCHS1, PstrCHS2 (*P. strobus*), and PsylCHS (*P. sylvestris*). Only PstrCHS1 is printed fully; dots in the other proteins indicate sequence identity. Arrows: active site of the condensing reaction (Cys169) (*22*); the Val instead of Phe in position 170 of PstrCHS2; the restriction sites used for the construction of protein hybrids between PstrCHS1 and PstrCHS2.

Table 2: Activities of the CHS1 from *P. strobus* (PstrCHS1) and the CHS from *P. sylvestris* (PsylCHS) with Different Starter Substrates

	relative activity (%)	
substrate	PstrCHS1	PsylCHS
4-coumaroyl-CoA	100	100
cinnamoyl-CoA	80 ± 20	60 ± 20
caffeoyl-CoA	50 ± 16	30 ± 14
dihydrocinnamoyl-CoA	6 ± 3	10 ± 4
feruloyl-CoA	<1	<1

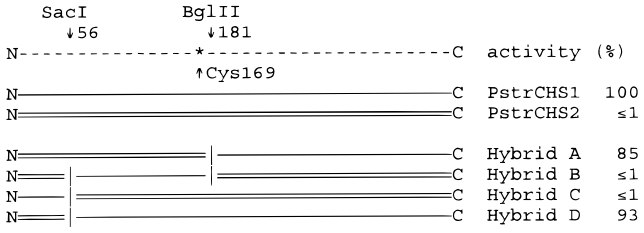

FIGURE 3: Protein hybrids of PstrCHS1 and PstrCHS2 and their activities with 4-coumaroyl-CoA as starter substrates. The positions of the shared restriction sites used for constructing the hybrids and the position of the active site of the condensing reaction (Cys169) (*22*) are indicated. The numbers refer to the protein sequence (Figure 2).

hybrid A contained PstrCHS2 up to amino acid 181. It possessed enzyme activity, indicating that the Val in position 170 was not responsible for the inactivity of PstrCHS2. The results with the other constructs suggested that the lack of activity of PstrCHS2 was based on sequences downstream of position 181, but the differences to PstrCHS1 and the CHS consensus provided no obvious clues for explanations.

In view of the functional diversity of CHS-related proteins (reviewed in ref *2*) it appeared possible that PstrCHS2 was not involved in chalcone biosynthesis, but served as a condensing enzyme in a pathway to other secondary plant products. However, assays with several candidate starter substrates (propionyl-CoA, butyryl-CoA, valeroyl-CoA, hexanoyl-CoA, heptanoyl-CoA, octanoyl-CoA, benzoyl-CoA, or crotonyl-CoA) failed to detect enzyme activity. This left the possibility that PstrCHS2 performed a so far unknown reaction in the biosynthesis of unusual chalcones. *P. strobus* indeed contains four chalcone derivatives which are unusual because they are C-methylated (**VII**−**X**, Figure 4) (*24*). Plant O-methyltransferases are well-known (*25*), but enzymes for C-methylation have not been described. Methyl side groups in other polyketides are introduced by the use of methylmalonyl-CoA as chain extender (*26*). The C-methylation in the *P. strobus* flavonoids is at the aromatic ring synthesized de novo by CHS. It was therefore an interesting possibility that it resulted from CHS activities that used cinnamoyl-CoA (**I**) and condensations with one methylmalonyl-CoA and two malonyl-CoA, as proposed in Figure 4. However, incubations with cinnamoyl-CoA and both labeled malonyl-CoA and methylmalonyl-CoA did not lead to a detectable product. The enzyme was purified to exclude the possibility that the labile methylmalonyl-CoA was quickly degraded or metabolized by other reactions in the crude extracts, but no activity was detectable.

PstrCHS2 Accepts a Derivative of the Diketide Intermediate in the CHS Reaction and Performs a Single Condensation Reaction with Methylmalonyl-CoA. The reaction sequence proposed for the formation of C-methylated chalcones would require that a single condensing enzyme accepted different chain extenders at a specific step in the three sequential condensation reactions. No such programming is known from CHS-type proteins or condensing enzymes in other polyketide synthases (*26*). It seemed therefore possible that PstrCHS2 did not perform all three condensations, but only the one with methylmalonyl-CoA. The positions of the C-methyl group (C6 and C8) seemed to suggest that this occurred either in the second or in the third condensation [at the diketide (**II**) or the triketide (**III**) level, Figure 4]. However, the chalcones can exist in isomeric forms (**V**, **VI**), and the position of the methyl group is fixed only after the isomerization of the chalcone to the flavanone by the action of chalcone isomerase (CHI). This suggested that a condensation with methylmalonyl-CoA at the level of a single intermediate could explain the formation of all four C-methylated flavonoids. The metabolite flow in flavonoid biosynthesis is channeled (*27, 28*), and a close association of CHS and the chalcone isomerase is particularly important to obtain the correct (−)-(2S)-isomer of the flavanone because only this form is used in the subsequent reactions (*1*). The use of methylmalonyl-CoA in the second condensation reaction leads to the chalcone configuration (**V**) that directly corresponds to that in the majority of the C-methylated flavonoids (**VII**, **IX**, **X**). The pathway in Figure 4 therefore proposes the diketide intermediate (**II**) as substrate for the condensation with methylmalonyl-CoA, but the alternative possibility at the level of (**III**) is not rigorously excluded. The figure also shows the subsequent enzymatic reactions to the C-methylated substances identified in *P. strobus*. If the enzyme could indeed perform only the single

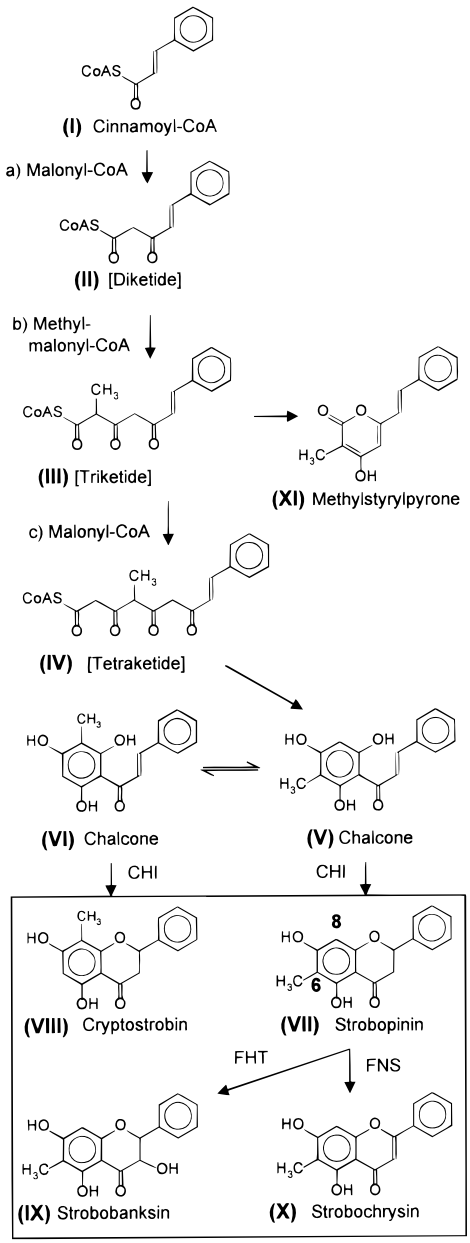

FIGURE 4: C-methylated flavonoids in *P. strobus* and pathway proposed for their biosynthesis. The chalcone backbone is synthesized in reactions that involve cinnamoyl-CoA (**I**) as starter and the sequential use of malonyl-CoA, methylmalonyl-CoA, and malonyl-CoA as chain extenders. The CHS products [methylated chalcones (**V**, **VI**)] are isomerized by chalcone isomerase (CHI) to the flavanones strobopinin (**VII**) or cryptostrobin (**VIII**). The flavone strobochrysin (**X**) and the dihydroflavonole strobobanksin (**IX**) result from the activities of flavone synthase (FNS) and flavanone-3β-hydroxylase (FHT), respectively (*1*). The methylstyrylpyrone (**XI**) is the expected release product if the reaction terminates at the triketide level (**III**).

condensation reaction from the diketide (**II**) to the triketide (**III**), the expected release product would be the methylstyrylpyrone (**XI**, 3-methyl-6-styryl-4-hydroxy-2-pyrone) (Figure 4), corresponding to the styrylpyrones observed with CHS reactions with phenylpropanoyl-CoA esters that terminated after two condensations with malonyl-CoA (*29*−*32*).

We used for these experiments the purified enzyme and a synthesized diketide derivative that corresponded to **II**, but with the CoA-moiety replaced by *N*-acetyl-cysteamine

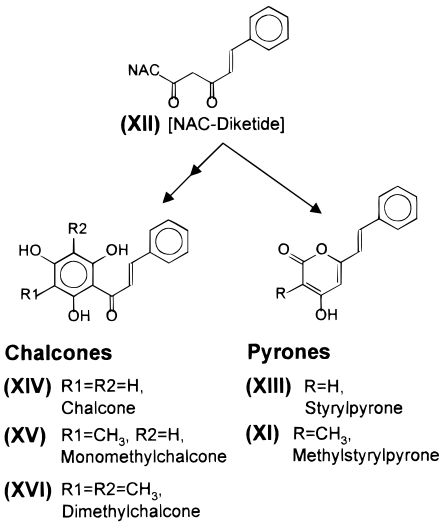

FIGURE 5: NAC-diketide derivative and pyrone products (one condensation reaction) and chalcone products (two condensation reactions) possible after chain extension with either methylmalonyl-CoA or/and malonyl-CoA.

(NAC-diketide, **XII**, Figure 5). The TLC analysis from incubations with radioactive methylmalonyl-CoA under the standard assay conditions (pH 7, 37 °C) revealed a small amount of a product at $R_f = 0.25$. Several modifications of the assay conditions, mostly aimed at reducing the nonenzymatic degradation of the instable methylmalonyl-CoA, showed that pH 6 and a temperature of 30 °C led to a large improvement (about 10-fold), with up to 40% of the radioactive methylmalonyl-CoA incorporated into the product. The substance showed the strong light-blue fluorescence (350 nm) typical for styrylpyrone derivatives (*30, 31*). The intensity of the fluorescence corresponded to the amount of radioactive product. On the basis of this correlation, the assays could be performed with unlabeled methylmalonyl-CoA if no precise quantification was necessary. No further improvement in product formation was obtained at pH 5.5 or at 25 °C. The formation was dependent on the presence of both substrates, because no product was detected after incubations containing only the NAC-diketide or only methylmalonyl-CoA.

Figure 6A, an example from a series of experiments to be discussed later, shows a radioscan after TLC analysis of the product obtained with the NAC-diketide and ^{14}C-labeled methylmalonyl-CoA under the optimized assay conditions. The substance migrating to $R_f = 0.25$ was identified as 3-methyl-6-styryl-4-hydroxy-2-pyrone (**XI**, Figure 4) by LC/MS and GC-EIMS (the data are given in the Experimental Procedures), and by comparison with a synthesized reference substance. The dimethylated product (**XVI**, Figure 5) expected from an additional condensation reaction with methylmalonyl-CoA was detected only in traces, as investigated with the authentic reference compound. The result showed that PstrCHS2 accepted a diketide derivative as substrate and that it performed a single condensation with methylmalonyl-CoA. Because it was possible that the substitution of the CoA-moiety by NAC was responsible for the activity with the diketide derivative, we also tested the NAC-derivative of cinnamic acid, but the enzyme was inactive with that substrate. The specificity of the enzyme for diketides was tested with acetoacetyl-CoA and radioactive

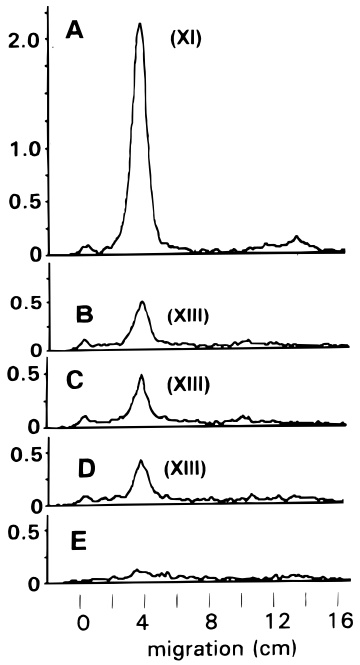

FIGURE 6: The activities of *P. strobus* CHS2 with the diketide NAC-derivative and various combinations of chain extenders. The panels show radioactivity scans after TLC separation of the products. The numbers at the ordinate indicate disintegrations per minute $\times 10^{-3}$. Chain extenders: (A) [^{14}C]methylmalonyl-CoA, (B) [^{14}C]malonyl-CoA, (C) unlabeled methylmalonyl-CoA plus [^{14}C]malonyl-CoA, (D) [^{14}C]methylmalonyl-CoA plus [^{14}C]malonyl-CoA, (E) [^{14}C]methylmalonyl-CoA plus unlabeled malonyl-CoA. Assay conditions: 20 μM NAC-diketide, 15 μM chain extender (88 000 dpm, when ^{14}C-labeled), 5 μg purified protein. The products were extracted at pH 9. The quantitative data are in Table 3, and the structures of the products are shown in Figure 5.

methylmalonyl-CoA. The activity was comparable to that obtained with the NAC-diketide, and the product was identified as 3,6-dimethyl-4-hydroxy-2-pyrone by GC-EIMS (data given in the Experimental Procedures), indicating that PstrCHS2 performed a single condensation also with acetoacetyl-CoA. Acetyl-CoA is a very poor substrate for typical CHS (*32*), and the efficient use of acetoacetyl-CoA therefore supported the notion that PstrCHS2 specifically accepted diketide derivatives. The pyrone or derivatives from it are not known from *P. strobus*, and the in vitro reaction with acetoacetyl-CoA probably has no physiological equivalent.

PstrCHS2 Does Not Complete the Reaction to Chalcones with the Diketide Derivative as Substrate. The high activity with the diketide derivative suggested that it might be able to synthesize a methylated chalcone with that substrate. This was investigated under the assay conditions optimized for the NAC-diketide. The TLC radioscans are shown in Figure 6, and Table 3 contains the quantitative data. The TLC solvent was chosen to separate the pyrone derivatives (R_f = 0.25) and flavanones (R_f = 0.4, produced by the chemical isomerization of the initial chalcone products under the assay conditions, see Experimental Procedures). Methylated and nonmethylated forms were not resolved under these conditions, and it should be noted that the experiments described below were designed to detect only radioactive products.

The activity with the NAC-diketide and methylmalonyl-CoA (Figure 6A) and the identification of the product as pyrone have been discussed above. Incubations with radioactive malonyl-CoA showed that this chain extender was also

Table 3: Quantification of the Data Obtained with the NAC-Diketides (Figures 6 and 7). The Assay Conditions are Given in the Legends to the figures. PsylCHS with Cinnamoyl-CoA and [^{14}C]Malonyl-CoA (Figure 7E): 10 800 dpm

chain extender		product (R_f)	
methyl-malonyl-CoA	malonyl-CoA	pyrone (0.25) (dpm)	chalcone (0.4) (dpm)
	PstrCHS2		
labeled		12 240	<50
	labeled	2570	<50
unlabeled	labeled	2490	<50
labeled	labeled	2430	<50
labeled	unlabeled	<80	<50
	PsylCHS		
labeled		2150	<50
	labeled	<80	4100
labeled	labeled	<80	4200
labeled	unlabeled	<80	<80

accepted (Figure 6B), but the activity was about 5-fold lower than with methylmalonyl-CoA. The product migrated to the position of the pyrone derivatives, indicating that the enzyme performed only one condensation to synthesize compound **XIII** (Figure 5). The next experiments addressed the question whether the protein synthesized the monomethylated chalcone (**V**) proposed in Figure 2. However, incubations with unlabeled methylmalonyl-CoA and labeled malonyl-CoA (Figure 6C) or with both chain extenders in labeled form (Figure 6D) led to the same result as with only labeled malonyl-CoA (Figure 6B). The data indicated that the enzyme was not capable of completing the reaction to chalcones and that methylmalonyl-CoA was not used in the presence of malonyl-CoA. The latter conclusion was confirmed with incubations that contained labeled methylmalonyl-CoA and unlabeled malonyl-CoA, because no significant incorporation from the radioactive chain extender was detectable (Figure 6E). The low activity and the instability of methylmalonyl-CoA did not permit a more detailed kinetic analysis of the competition between the two chain extenders.

Comparison of PstrCHS2 with a CHS from a Plant Not Containing C-Methylated Flavonoids. Both the NAC-diketide and methylmalonyl-CoA had not been tested before with CHS. Therefore, we investigated with a typical CHS whether the enzyme had the same properties as PstrCHS2. We chose the CHS from *Pinus sylvestris* L. (Scots pine) because this plant does not contain C-methylated flavonoids (*24*), and because PsylCHS is closely related to PstrCHS2 (86.8% identity, see Figure 2). All assays were performed with the enzyme purified after expression in *E. coli*. The material remaining at the origin of the TLC chromatograms from some of these assays is unknown; it may represent polymerized products.

Figure 7A shows that the enzyme accepted the NAC-diketide to synthesize with labeled methylmalonyl-CoA a radioactive product migrating to the position of the methylstyrylpyrone (R_f = 0.25, **XI** in Figure 5), and the substance showed the typical strong fluorescence at 350 nm. This indicated that the use of methylmalonyl-CoA was not a property unique to PstrCHS2, and that PsylCHS also performed only one condensation reaction with that chain extender. With malonyl-CoA (Figure 7B), however, it completed the reaction to the chalcone (**XIV**, Figure 5).

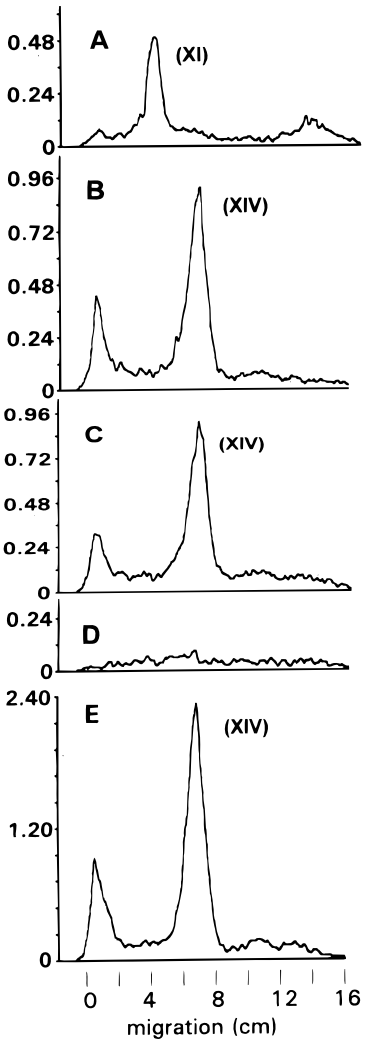

FIGURE 7: The activities of *P. sylvestris* CHS with the diketide NAC-derivative as starter substrate and various combinations of chain extenders. The panels show radioactivity scans after TLC separation of the products. The numbers at the ordinate indicate disintegrations per minute $\times 10^{-3}$. Chain extenders: (A) [^{14}C]-methylmalonyl-CoA, (B) [^{14}C]malonyl-CoA, (C) [^{14}C]Methylmalonyl-CoA plus [^{14}C]malonyl-CoA, (D) [^{14}C]methylmalonyl-CoA plus unlabeled malonyl-CoA. Panel E shows the activity with cinnamoyl-CoA and [^{14}C]malonyl-CoA. The quantitative data are in Table 3, and the structures of the products are shown in Figure 5. The assay conditions were as described in the legend to Figure 6, except that the incubations contained 1 μg purified PsylCHS.

Although the incorporation of radioactivity was about 2-fold higher in Figure 7B than in Figure 7A, the results were comparable in terms of nanomoles of synthesized product because the chalcone formation required two chain extenders while only one was incorporated into the methylstyrylpyrone. The data suggested that PsylCHS might be capable of synthesizing methylated chalcones in vitro, although they are not present in the plant. Experiments with both radioactive chain extenders indeed led to a product migrating to the position of the chalcones (Figure 7C), but the rate of incorporation of radioactivity was the same as with labeled malonyl-CoA alone (Table 3). This suggested that PsylCHS, like PstrCHS2, did not use methylmalonyl-CoA in the presence of malonyl-CoA. We therefore tested radioactive methylmalonyl-CoA plus unlabeled malonyl-CoA, and the result in Figure 7D showed indeed no significant formation of a radioactive product, confirming that methylmalonyl-CoA was not used under these conditions.

The results indicated that PsylCHS was similar to PstrCHS2 in two properties: it performed only one condensation with methylmalonyl-CoA, and this activity was suppressed in the presence of malonyl-CoA. The two enzymes were clearly different in their activities with malonyl-CoA, because PsylCHS performed two condensation reactions to synthesize the chalcone, while PstrCHS2 performed only one condensation and produced the styrylpyrone. The combination NAC-diketide plus malonyl-CoA was also tested with CHS cloned from other plants and with stilbene synthases. In all cases the reactions proceeded to the chalcones or stilbenes, respectively (unpublished results).

PsylCHS also allowed a comparison of the efficiency with which the NAC-diketide and cinnamoyl-CoA were accepted as substrates. Figure 7E shows the activity with cinnamoyl-CoA plus radioactive malonyl-CoA. The comparison with Figure 7D (note the different scales) suggested at first sight that the NAC-diketide was accepted much less efficiently than cinnamoyl-CoA. However, the synthesis of the chalcone from cinnamoyl-CoA used three radioactive malonyl-CoAs, while only two were required with the NAC-diketide. The calculation in terms of nanomoles of product indicated that the NAC-diketide was accepted with 60−70% efficiency when compared to cinnamoyl-CoA. We also tested with PsylCHS the NAC-derivative of cinnamic acid that was no substrate for PstrCHS2. It was accepted as a starter substrate, with efficiencies of about 60% when compared with cinnamoyl-CoA.

Biosynthesis of C-Methylated Chalcones: A Working Hypothesis. The properties of PstrCHS2 are unique in the family of CHS-related enzymes. A similar specialization is not known from any other of these polyketide synthases, and there is no need for it in the standard CHS reaction which uses malonyl-CoA. This could be different, however, if the product formation also required the use of methylmalonyl-CoA. The available data indicate that these simplest polyketide synthases are not capable of performing a reaction sequence requiring the sequential use of two different chain extenders. We propose that PstrCHS2 specifically contributes with methylmalonyl-CoA the condensation reaction from the diketide to the triketide level in the biosynthesis of C-methylated chalcones (Figure 4).

The data indicate that the complete reaction sequence cannot be performed by a single homodimeric protein. Previous results (*21*) showed that each subunit of standard CHS can perform all three condensation reactions, and this argues against a heterodimer as one of the possibilities. We therefore propose an interaction of two different homodimers in a complex that provides metabolite channeling (*27, 28*) and also an environment in which only methylmalonyl-CoA is supplied for the PstrCHS2 function. The model predicts that the diketide and triketide intermediates are exchanged between two interacting proteins. The release of derailment products after the first condensation are well-known from CHS, suggesting that the intermediates are not buried deeply in the protein. Our results show that PstrCHS2 and other CHSs accepted a diketide derivative as substrate. Other data suggest that the subsequent reaction intermediates are accessible at the surface of the protein. The most convincing evidence is from the biosynthesis of reduced chalcones (6′-

deoxychalcones) which requires the reduction of an enzyme-bound CHS intermediate at the tri- or tetraketide level by a reductase interacting with CHS (*33*, *34*). Taken together, the data suggest that an exchange of intermediates between two CHS-type proteins is possible.

The PstrCHS2 product in vitro was a 4-hydroxy-2-pyrone derivative. In the model of cooperating enzymes, it represents a derailment product resulting from the lack of the partner accepting the triketide intermediate to perform the last condensation reaction. There are several examples where pyrones were identified as release products from polyketide synthase reactions terminating at the triketide stage. With CHS, they were detected as byproducts of in vitro reactions (*29*−*32*). Other well-known examples are the fungal methylsalicylic acid synthase and the plant 6-hydroxymellein synthase that use acetyl-CoA and malonyl-CoA. These enzymes release 6-methyl-4-hydroxy-2-pyrone as derailment product if the reduction step necessary at the triketide stage is blocked (*35*−*38*). Perhaps even more interesting in the context of cooperating proteins are the modular polyketide synthases, e.g., the 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, which consists of three polypeptides. DEBS1 contains two modules which catalyze the first two rounds of chain extension, and 4-hydroxy-2-pyrone derivatives are released if only DEBS1 is active (*39*−*42*). Experiments with NAC-derivatives corresponding to the product of the first condensation reaction also showed that the diketide is recognized by the condensing enzyme of the second module (*43*). It could be argued that the biosynthesis of C-methylated chalcones represents the simplest case of a modular polyketide synthase reaction.

Finally, it should be noted that the model proposed here may be of general interest, because C-methylated flavonoids are not confined to *P. strobus*. Although they are not common, more than 50 different C-methylated flavonoids are known from other plants (*44*−*48*). Interestingly, some plants contain 6,8-dimethylated chalcone derivatives. According to the model proposed for *P. strobus*, the biosynthesis would require that methylmalonyl-CoA is used not only for the second, but also for the third condensation. The CHS in these plants have not yet been analyzed, but it would be interesting to investigate whether they contain enzyme variants with properties that could explain the biosynthesis of dimethylated chalcones.

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