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Stilbenecarboxylate biosynthesis: a new function in the family of chalcone synthase-related proteins

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Dedicated to Meinhart H. Zenk on the occasion of this 70th birthday

Abstract

Chalcone (CHS), stilbene (STS) synthases, and related proteins are key enzymes in the biosynthesis of many secondary plant products. Precursor feeding studies and mechanistic rationalization suggest that stilbenecarboxylates might also be synthesized by plant type III polyketide synthases; however, the enzyme activity leading to retention of the carboxyl moiety in a stilbene backbone has not yet been demonstrated. *Hydrangea macrophylla* L. (Garden Hortensia) contains stilbenecarboxylates (hydrangeic acid and lunularic acid) that are derived from 4-coumaroyl and dihydro-4-coumaroyl starter residues, respectively. We used homology-based techniques to clone CHS-related sequences, and the enzyme functions were investigated with recombinant proteins. Sequences for two proteins were obtained. One was identified as CHS. The other shared 65–70% identity with CHSs and other family members. The purified recombinant protein had stilbenecarboxylate synthase (STCS) activity with dihydro-4-coumaroyl-CoA, but not with 4-coumaroyl-CoA or other substrates. We propose that the enzyme is involved in the biosynthesis of lunularic acid. It is the first example of a STS-type reaction that does not lose the terminal carboxyl group during the ring folding to the end product. Comparisons with CHS, STS, and a pyrone synthase showed that it is the only enzyme exerting a tight control over decarboxylation reactions. The protein contains unusual residues in positions highly conserved in other CHS-related proteins, and mutagenesis studies suggest that they are important for the structure or/and the catalytic activity. The formation of the natural products in vivo requires a reducing step, and we discuss the possibility that the absence of a reductase in the in vitro reactions may be responsible for the failure to obtain stilbenecarboxylates from substrates like 4-coumaroyl-CoA.

Keywords: *Hydrangea macrophylla*; Garden Hortensia; Homology based DNA cloning; Chalcone synthase; Stilbene synthase; Stilbenecarboxylate synthase; Recombinant protein expression; Hydrangeic acid; Phyllodulcin; Lunularic acid

1. Introduction

Chalcone synthase (CHS) is a plant-specific polyketide synthase that uses a starter CoA-ester from the phenylpropanoid pathway as substrate, performs three sequential condensation reactions with malonyl-CoA, and folds the resulting tetraketide intermediate to a new aromatic ring system (chalcone). It is the prototype for a family of enzymes that synthesize a surprisingly large variety of products. The diversity is based on the use of different starter CoA-esters (e.g. phenylpropanoids, benzoic acids, or aliphatic acids), different number of condensation reactions (one, two, or three), and different types of ring folding after three condensations (Schröder, 2000). Fig. 1 shows as example the products that have been obtained from 4-coumaroyl-CoA. CHS-related sequences have also been identified in bacteria, but the biochemical functions are known in only two

Abbreviations: 2PS, 2-pyrone synthase; CTAL, 4-coumaroyltriacetic acid lactone; CHS, chalcone synthase; STS, stilbene synthase; STCS, stilbenecarboxylate synthase.

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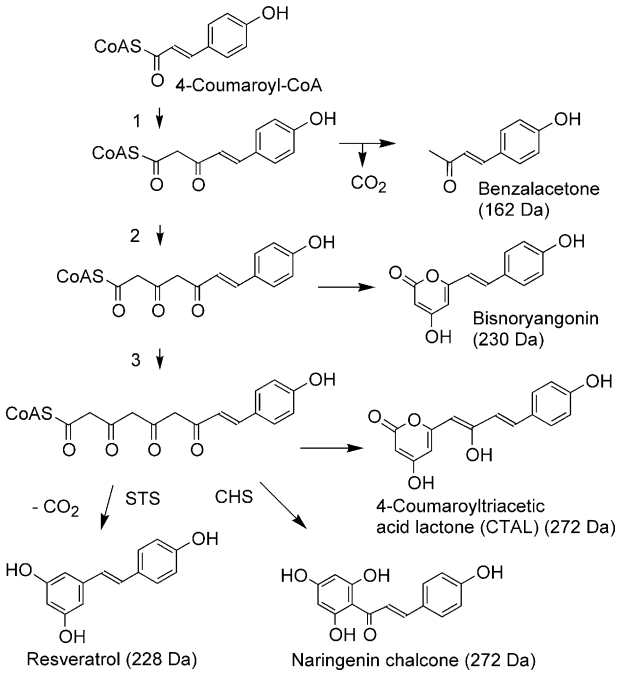

Fig. 1. Compounds synthesized from 4-coumaroyl-CoA by CHS and related enzymes. Benzalacetone is obtained from one condensation followed by decarboxylation (Borejsza-Wysocki and Hrazdina, 1996; Abe et al., 2001). Bisnoryangonin is a release product after two condensations. CHS and STS perform three condensations, but the ring folding is different (CHS: Claisen condensation; STS: aldol condensation). 4-Coumaroyltriacetic acid lactone (CTAL) (Akiyama et al., 1999) is obtained after three condensation reactions in absence of the ring folding to chalcone or stilbene.

cases (Funa et al., 1999, 2002; Pfeifer et al., 2001; Chen et al., 2001).

The recently published structure of a CHS (Ferrer et al., 1999) identified key elements of the CHS reaction, and this now permits predictions on the residues that control the substrate specificity and the number of condensation reactions in other enzymes of the family. The value of such analyses was demonstrated with a pyrone synthase (2PS) from *Gerbera hybrida* (Eckermann et al., 1998) that uses acetyl-CoA and two condensation reactions to synthesize methylpyrone (triacetic acid lactone), the precursor of a phytoalexin in that plant. The crystal structure of 2PS confirmed the postulated small size of the active site pocket, and site-directed mutagenesis based on the structural elucidation showed that three amino acid exchanges were sufficient to change a CHS into a pyrone synthase (Jez et al., 2000a). However, such predictions have been difficult with some other enzymes of the protein family, and one example is the difference between CHSs and stilbene synthases (STSs). Like CHSs, STSs utilize phenylpropanoid substrates and perform three condensations, but the ring folding is different (aldol condensation versus Claisen condensation), and the terminal carboxyl group is removed in the product (Fig. 1). All STSs share 60–70% identity with CHSs, but the various STSs are a rather heterogeneous group having only 60–70% identity to each other. STSs probably evolved several times independently from CHSs (Tropf et al., 1994), and it seems likely that the change to the STS-type ring folding can be achieved by several different alterations of CHSs. Modelling of STSs based on the CHS structure did not provide decisive clues on the mechanistic differences.

The STS reaction is of particular interest because a similar activity can be predicted for the biosynthesis of stilbenecarboxylic acids. These substances and their derivatives are abundant in liverworts, but they also occur in some higher plants (Gorham, 1995). Some of them have interesting properties, e.g. phyllodulcin is a substance 600- to 800-fold sweeter than sucrose (Suzuki et al., 1978). Precursor feeding studies indicated that the backbone is synthesized via the shikimate/malonate route like chalcones and stilbenes (Billek and Kindl, 1962; Ibrahim and Towers, 1962; Pryce, 1971). The enzyme reaction should involve a STS-type ring folding without removal of the terminal carboxyl group (Fig. 2), but such stilbenecarboxylate synthase (STCS) activities have never been demonstrated in vitro. The formation of the natural products also requires a reducing step, but it is unknown at which level it occurs. We became interested in these predicted polyketide synthase reactions because the assignment to the family of CHS-related enzymes would add a new reaction type performed by these proteins, and as a consequence provide possibilities to synthesize new products. It was also hoped that these enzymes would provide new insights into the differences between CHSs and STSs. The Garden Hortensia (*Hydrangea macrophylla*) was chosen for the experiments because it contains a simple set of stilbenecarboxylates and derivatives that could be explained by a key STCS reaction with 4-coumaroyl-CoA or dihydro-4-coumaroyl-CoA (Fig. 2). We used a homology-based PCR strategy to obtain genes and cDNAs for CHS and related sequences, and the enzyme functions were investigated with recombinant proteins.

2. Results

2.1. CHS and related sequences in H. macrophylla, and the activities of recombinant proteins with 4-coumaroyl-CoA

The first experiments were carried out by PCR reactions with genomic DNA and degenerate primers designed from the conserved region around the active site cysteine (sense 1) and the conserved region at the *C*-terminal of the protein (antisense). PCR products of the expected size (650 bp) were cloned and a representative example was sequenced. It revealed 75–80% identity at the DNA and ca. 90% identity at the protein level with CHS sequences from various plants. The

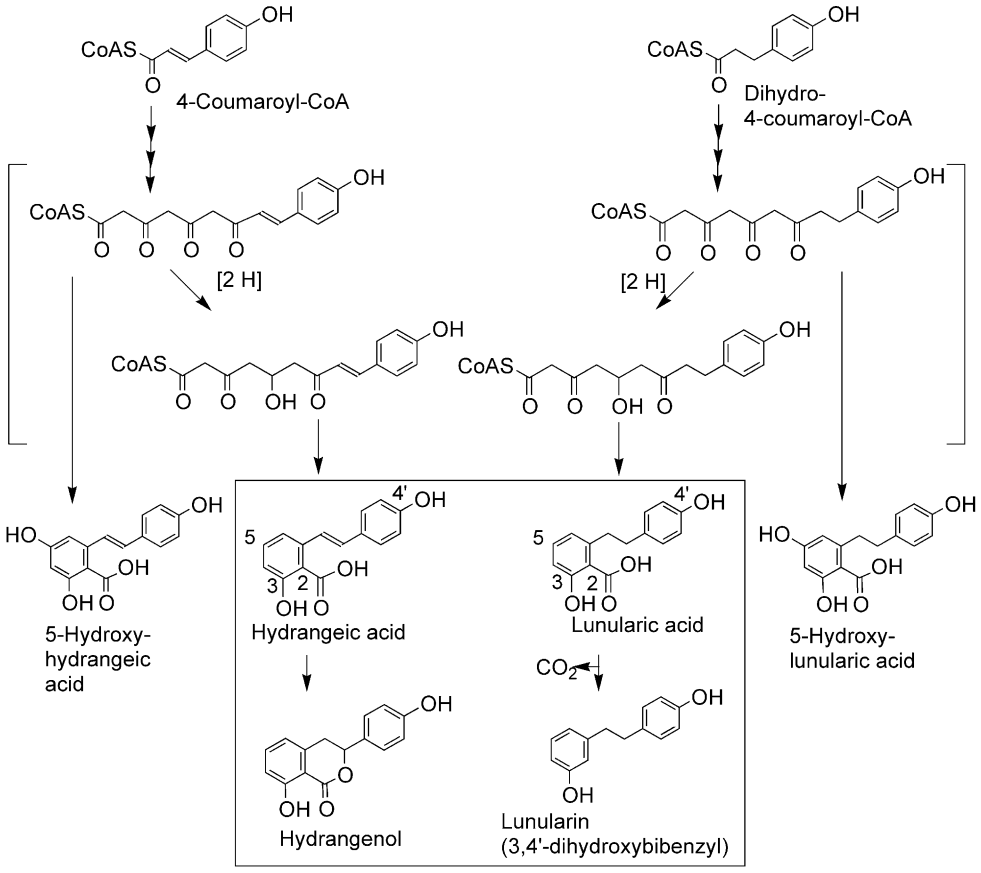

Fig. 2. Stilbenecarboxylates and derivatives in *H. macrophylla*, and the proposed biosynthesis. The box shows the natural products identified from the plant. The formation of the backbones can be explained by an STS-type reaction with 4-coumaroyl-CoA or dihydro-4-coumaroyl-CoA that retains the terminal carboxyl group (stilbenecarboxylate synthase, STCS). The biosynthesis of the natural product includes a reducing step. It is unknown at which level this occurs, and the intermediates proposed here (in the bracket) are hypothetical. 5-Hydroxyhydrangeic acid and 5-hydroxylunularic acid are the STCS products expected in absence of the reducing step.

labelled DNA fragment was then used to screen a library of genomic DNA. The screen yielded three phages containing hybridizing sequences. Two of them contained genes with incomplete or corrupted coding regions (not shown), and the third contained the complete gene (*HmCHS*) belonging to the PCR fragment. The function of the protein was tested after excision of the intron by mutagenesis and protein expression in *Escherichia coli*. The enzyme synthesized naringenin chalcone from 4-coumaroyl-CoA, and thus was identified as a CHS.

DNAs for other CHS-related proteins were obtained by PCR with a newly designed degenerate sense primer (sense 2). The results identified a 630 bp genomic fragment that shared at the DNA level 60–65% identity with various CHSs (including that described here from *H. macrophylla*) and CHS-related genes, and ca. 70% identity at the level of the predicted amino acid sequence. Various combinations of additional primers and 5′- and 3′-RACE techniques were used to obtain the complete gene and cDNA. The cDNA and the gene were named *HmSTCS1a* and *HmSTCS1b*, respectively, based on results described later in this report. They were very similar, with only seven amino acid differences in the deduced proteins. None of them was in positions known to be important for substrate specificities of CHS and CHS-related proteins (Ferrer et al., 1999; Jez et al., 2000a). It seems likely that *HmSTCS1a* and *HmSTCS1b* are alleles of the same gene.

The protein encoded by the cDNA was expressed in *E. coli* and purified (STCS1a). The first assays were carried out with 4-coumaroyl-CoA, to see whether it synthesized the CHS product naringenin chalcone, or resveratrol, the stilbene expected from a STS activity. The TLC analysis revealed two radioactive substances, but none of them migrated to the position of naringenin or resveratrol. The further analysis showed that one of the two products was bisnoryangonin (Fig. 1), and the identification was confirmed by LC–MS and comparison with the authentic substance (not shown). The LC–MS analysis of the second product determined a mass of 272 Da. This would be expected for 5-hydroxyhydrangeic acid synthesized by a STCS reaction (Fig. 2), but also for 4-coumaroyltriacetic acid lactone (CTAL), the pyrone product after three condensations without CHS- or STS-type ring folding (Fig. 1). The LC–MS/

MS analysis showed a base peak ion at m/z 147 that is typical for the coumaroyl moiety ($[HOC_6H_4CH{=}CH{-}C{=}O]^+$). Comparison of the structures showed that it could be derived only from CTAL, not from 5-hydroxyhydrangeic acid, and this indicated that the enzyme did not synthesize a stilbenecarboxylic acid from 4-coumaroyl-CoA. A further characterization of the enzyme activity revealed a broad temperature maximum around 30 °C, slightly different pH optima for the two products (pH 6.5 for CTAL, pH 7 for bisnoryangonin), a product ratio of ca. 6 to 1 in favor of CTAL, and an apparent K_m of 55–60 μM for 4-coumaroyl-CoA (calculated from the sum of the two radioactive products).

While this work was in progress, cDNAs for a CHS and a CHS-related protein from another *Hydrangea* variety, *H. macrophylla* var. *thunbergii*, were reported (Akiyama et al., 1999). The CHS cDNA differed in the coding region in more than 20 positions from the *HmCHS* gene isolated in our work, and this resulted in seven amino acid exchanges (Fig. 3). The recombinant protein was also identified as CHS by the synthesis of naringenin chalcone from 4-coumaroyl-CoA. The gene *HmSTCS1b* was very similar to the CHS-related cDNA from the variety *thunbergii* (two amino acid differences), but the cDNA *HmSTCS1a* predicted five differences in the deduced protein (Fig. 3). Like STCS1a, the recombinant protein from the variety *thunbergii* also synthesized only CTAL and bisnoryangonin from 4-coumaroyl-CoA. This plant contains the natural product hydramacroside B (Yoshikawa et al., 1994b) that is probably derived from a linear tetraketide (Fig. 4). It was suggested that the CHS-related protein provided such precursor, and CTAL was proposed to be a derailment product in absence of the subsequent reactions (Akiyama et al., 1999).

Taken together, these independent findings from different approaches suggested that *Hydrangea* expressed two polyketide synthases of the CHS-type protein

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a) H. macrophylla CHS gene                      MVTVEEVRKAQRAEGPATILAIGTATPPNYVDQS
b) H. m. var. thunbergii CHS cDNA               .................................
c) H. macrophylla STCS1a cDNA                 MATKSVAVEEMCKAQKAGGPATILAIGTAVPSNCYYQS
d) H. macrophylla STCS1b gene                 .....................................
e) H. m. var. thunbergii CHS-related          .....................................

a) TYPDFYFRVTNSEHKKELKAKFQRMCNNSQIKKCYMHLTEEILKLNPNICAYMVPSLDARQDMVVVEIPKLGKEAA
b) ........................D......R..........E........A.....................
c) EYPDFYFRVTKSDHLTDLKSKFKRMCDRSSIKKRYMHLTEEILKENPNMCSFAAPSIDGRQDIVVKEIPKLAKEAA
d) ........................E....T...........E......T.........................
e) ........................E.............E......T.........................

                                                        164C▼165 ▼170
a) TRAIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAENNKGARV
b) ..................................................................
c) SKAIKEWGQPESNITHLVFCTTSGVDMPGCDYQLTRLLGLRPSIKRLMMYQQGCHAGGTGLRLAKDLAENNKGARV
d) ..........K.....................................................................
e) ..........K.....................................................................

a) LVVCSEITAVTFRGPSDTHLDSLVGQALFGDGARAVIIGSDPMPEVEKPLFEIVSAAQTILPDSDGAIDGHLREVG
b) ................................A.....................................
c) LVVCSEMTVINFRGPSEAHMDSLVGQSLFGDGASAVIVGSDPDLSTEHPLYQIMSASQIIVADSEGVIDGHLRQEG
d) ..............................................................A.....
e) ..............................................................A.....

                                  ▼▼▼▼▼▼          H303
a) LTFHLLKDVPGLISKNIEKSLVEAFRPL------DISDWNSIFWIAHPGGPAILDQVEKKLALKPEKLRATRNVLS
b) ..............................------.....................................
c) LTFHLRKDVPSLVSDNIENTLVEAFTPILMDSIDSIIDWNSIFWIAHPGGPAILNQVQAKVGLKEEKLRVSRHILS
d) ..................................................................
e) ..................................................................

      N336
a) DYGNMSSACVLFIMDEMRKNSVYEGLMTTGEGLEWGVLFGFGPGLTVETVVLHGVST  389
b) ....................AE.................................  389
c) EYGNMSSACVFFIMDEMRKRSVEEGKGTTGEGLEWGVLFGFGPGFTVETIVLHSVPI  399
d) ....................M.................................  399
e) ..................................................  399
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Fig. 3. Deduced amino acid sequences of the enzymes from *Hydrangea*. The data for the var. *thunbergii* are from reference (Akiyama et al., 1999) (GenBank accessions AB011467 and AB011468). Boxed (numbering according to the crystallized CHS from *Medicago sativa* (Ferrer et al., 1999)): active site (C164); residues mainly involved in the decarboxylation of malonyl-CoA (H303, N336). The nucleotide sequence data from the present work are available from the GenBank database with the accession numbers AF456445 and AF456446 (*HmSTCS*), and AF456447 and AF456448 (*HmCHS*).

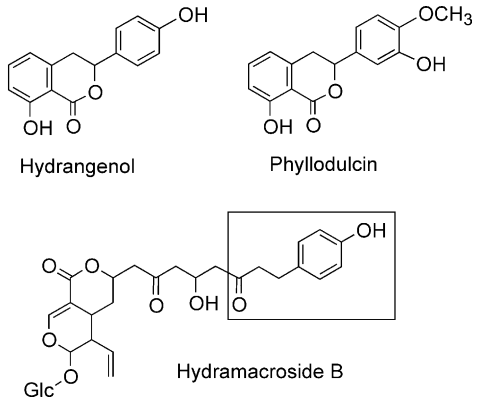

Fig. 4. Natural products from *H. macrophylla* var. *thunbergii*. Dominant stilbenecarboxylic acid derivatives (hydrangenol and phyllodulcin), and hydramacroside B, a secoiridoid derivative that is presumably derived from a linear tetraketide synthesized by a polyketide synthase. Glc, glucose. The box indicates the dihydro-4-coumaroyl residue that is the likely starter for the polyketide synthase reaction.

family: a CHS as expected for flavonoid biosynthesis, and an enzyme that performed two or three condensations and released the corresponding lactones. The data available from 4-coumaroyl-CoA suggested that the CHS-related enzymes from the two varieties were identical in their functions.

These results were unexpected and unsatisfactory for at least two reasons. The first was the failure to detect the enzyme(s) for stilbenecarboxylate biosynthesis. The second was that this simple picture did not seem to reflect the pronounced differences between the two *Hydrangea* varieties. The variety *thunbergii* does not only look different from the European Garden Hortensia *H. macrophylla*, but the spectrum of natural products is also different. Both contain 4-coumaroyl-CoA derived stilbenecarboxylates and their derivatives (e.g. hydrangeic acid and hydrangenol, Fig. 2), but several other compounds appear to be present in only one of the two varieties. Lunularic acid and lunularin (derived from dihydro-4-coumaroyl-CoA, Fig. 2) were described only from *H. macrophylla*, while the variety *thunbergii* was found to contain several substances presumably originating from caffeoyl-CoA (e.g. phyllodulcin, Fig. 4, and several other compounds) (Yoshikawa et al., 1992, 1994a, 1996a,b, 1999). Moreover, hydramacrosides have been reported only from the variety *thunbergii*, not from the Garden Hortensia (*H. macrophylla*), and therefore it was not obvious that the explanation for the CHS-related enzyme could be extended to other varieties. One possible explanation was that the STCS reaction is carried out by other polyketide synthases, or by CHS-related proteins that were not detected by the homology-based approaches. However, it also seemed possible that a STCS activity of the CHS-related protein was not detected because it required a different substrate (only 4-coumaroyl-CoA had been tested so far), or because the in vitro incubations lacked some condition that was necessary for the correct function of the protein.

2.2. STCS1a synthesizes a stilbenecarboxylate from dihydro-4-coumaroyl-CoA

H. macrophylla contains lunularic acid and its decarboxylation product lunularin, and dihydro-4-coumaroyl-CoA should be the precursor (Fig. 2). Experiments with this substrate showed incorporation rates of labelled malonyl-CoA into radioactive products that were 50–80% higher than with 4-coumaroyl-CoA, suggesting that dihydro-4-coumaroyl-CoA was a better substrate than 4-coumaroyl-CoA. Fig. 5A summarizes the products that could be expected, taking into account the conclusions from the experiments with 4-coumaroyl-CoA that the enzyme performed two or three condensations, but was not a CHS or STS. The figure also indicates the likely fragmentation patterns of the products.

The TLC analysis with several solvents and different types of carrier materials (cellulose and RP18) showed that the enzyme synthesized at least two radioactive products. The analysis by positive and negative LC–MS identified one as dihydrobisnoryangonin (232 Da), the lactone release product after two condensations (Fig. 5A). This was confirmed by the positive ion CID spectrum which displayed fragments at m/z 107 (100%) and m/z 127 (10%). The latter fragment comprising the pyrone ring was also detected in the CID MS of dihydro-CTAL (see below and Fig. 5A). The second major product had a mass of 274 Da, as expected for both the stilbenecarboxylic acid (5-hydroxylunularic acid) and dihydro-CTAL. Although suitable reference samples were not available, the identification of dihydro-CTAL was possible in the CID spectrum because this compound but not 5-hydroxylunularic acid had a key ion at m/z 127. A similarly positive identification of the stilbenecarboxylic acid was not possible with these techniques.

However, a permethylation of the hydroxyl groups in both compounds resulted in different products. The GC–MS analysis showed that the main product displayed a M^+-ion at m/z 330 (8%) in its EIMS, which was consistent with the conclusion that the preparation contained permethylated 5-hydroxylunularic acid (Fig. 5B). Further key ions appeared at m/z 299 ($[M-CH_3O]^+$, 5), 298 ($[M-CH_3OH]^+$, 6), 190 (4), 148 (3), 121 ($CH_3O–C_6H_4–CH_2^+$, 100), 91 (5) and 77 (5). We also analyzed the methylated product of dihydrobisnoryangonin (232 Da) to test the methylation conditions. The product showed a M^+ ion at m/z 260 (10%) and significant fragments at m/z 134 (4), 125 (6), 121 ($CH_3O–C_6H_4–CH_2^+$, 100), 85 (8) and 69 (12), confirming that the pyrone ring had not been opened (Fig. 5B). The results supported the assumption that a methylation of dihydro-CTAL would not lead to a ring opening. Finally, the same experiment was done with

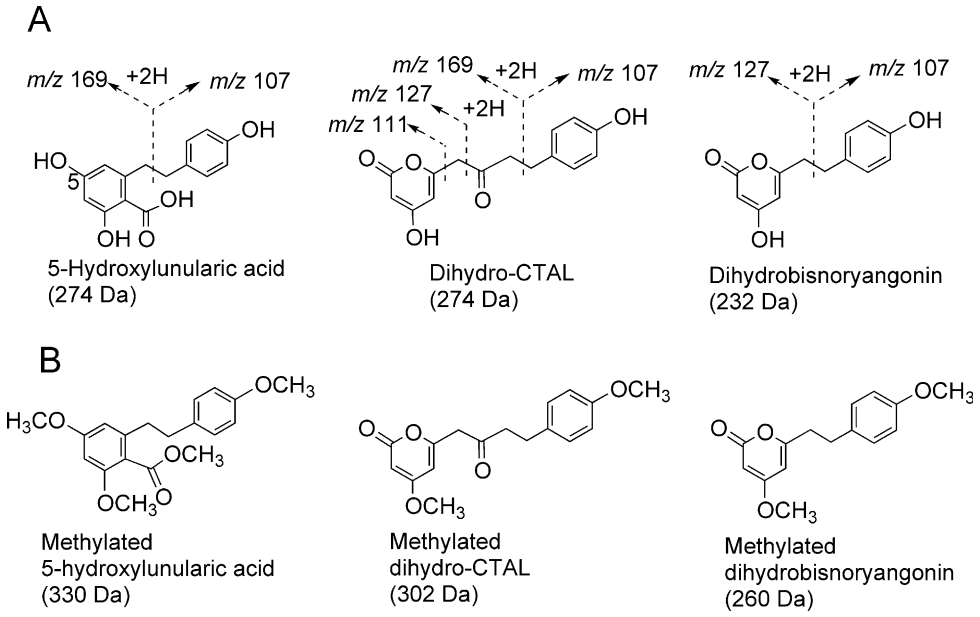

Fig. 5. Products synthesized from dihydro-4-coumaroyl-CoA by STCS1: (A) predicted fragmentation patterns from products of three condensation reactions (5-hydroxylunularic acid: stilbenecarboxylic acid; dihydro-CTAL: pyrone derailment in absence of STS- or CHS-type ring closure) and two condensation reactions (dihydrobisnoryangonin); (B) methylated products. The masses permit the distinction between the three substances.

lunularic acid (Fig. 2) as reference compound which is identical to 5-hydroxylunularic acid (Fig. 5A) except for the lack of the hydroxyl function at position 5. Permethylated lunularic acid showed a M^+ ion at m/z 300 (M^+, 15%). Fragment ions at m/z 269 ($[M-CH_3O]^+$, 6), 268 ($[M-CH_3OH]^+$, 7), 148 (8), 121 ($CH_3O-C_6H_4-CH_2^+$, 100), 105 (9), 91 (17) and 77 (22) indicated a similar fragmentation when compared with that of the proposed 5-hydroxylunularic acid synthesized from dihydro-4-coumaroyl CoA in vitro.

The EtOAc-extracted products from dihydro-4-coumaroyl-CoA were further analysed by ^{1}H NMR. The results indicated a mixture of three compounds each containing a 4-substituted phenyl ring (δ 6.7–6.8 and δ 7.0–7.1, J = 8–9 Hz). Further signals of the spectrum, showing two pairs of doublets at δ 5.28 and 5.88 and at δ 5.33 and 6.05 (J = 2.1 Hz), could be assigned to the protons H-3 and H-5 of two 4-hydroxy-2-pyrone rings and thus identified the second part of the structure in two compounds (dihydrobisnoryangonin and dihydro-CTAL). The third compound was further characterized by two doublets at δ 6.3 (J = 2.2 Hz) that could be assigned to a 2-carboxyl-3,5-dihydroxyphenyl ring system. Characterization of the enzyme products with HPLC indeed led to a fraction containing a substance characterized by signals that could be assigned to a 4-OH-substituted phenyl ring (δ 6.68 and 6.99), two methylene groups with the same chemical shift (δ 2.75, *brs*, 4H), and a 2-carboxyl-3,5-dihydroxyphenyl ring (δ 6.15 and 6.16). These data were consistent with the structure of 5-hydroxylunularic acid, the only product containing a 2-carboxyl-3,5-dihydroxyphenyl ring system. The results of the ^{1}H NMR analysis confirmed the other data that the CHS-related enzyme synthesized a stilbenecarboxylic acid from dihydro-4-coumaroyl-CoA. A rough quantification suggested that 5-hydroxylunularic acid represented ca. 40–45% of the product mixture, and that the rest was divided equally between dihydro-CTAL and dihydrobisnoryangonin. A further more detailed analysis of the stilbenecarboxylic acid was not possible because of the instability of the compound, a property of molecules containing a 2-carboxyl-3,5-dihydroxyphenyl residue that has been discussed many years ago (Gorham, 1980).

The further characterization of the activity with dihydro-4-coumaroyl-CoA revealed slightly different pH optima for the formation of 5-hydroxylunularic acid (pH 6) and dihydrobisnoryangonin (pH 7). The apparent K_m for dihydro-4-coumaroyl-CoA was 9-13 μM, and thus the affinity for this substrate was higher than for 4-coumaroyl-CoA. The enzyme revealed a broad temperature maximum around 30 °C.

2.3. Activities of STCS1a with other substrates

Other CoA-esters were tested to obtain an overview on the activities with other substrates and to investigate whether a STS-type ring folding with retention of the carboxyl group could also be performed with other substrates. No activity was detected with acetyl-CoA, crotonyl-CoA, and 3-hydroxybutyryl-CoA. Low activities (compared to dihydro-4-coumaroyl-CoA) were found with small aliphatic CoA-esters, e.g. propionyl-CoA, butyryl-CoA, or isovaleryl-CoA. Low activities were also observed with benzoyl-CoA, sinapoyl-CoA and caffeoyl-CoA. The latter is interesting because

caffeoyl-CoA could be the starter substrate for the biosynthesis of thunberginols and phyllodulcin (Fig. 4) which represent major stilbenecarboxylate derivatives in the variety *thunbergii*, but not in *H. macrophylla*. The TLC analysis indicated for all substrates at least two radioactive products, but they were not analyzed in detail.

A detailed product characterization was carried out with substrates revealing activities that were in the same range as with dihydro-4-coumaroyl-CoA or 4-coumaroyl-CoA. This was observed with longer aliphatic CoA-esters (hexanoyl-CoA, heptanoyl-CoA), phenacetyl-CoA, feruloyl-CoA, and cinnamoyl-CoA, indicating that large hydrophobic molecules were the preferred substrates in vitro. LC–MS analysis identified in all cases the lactones formed after two condensation reactions as one of the products. The lactones after three condensation reactions were the main products from cinnamoyl-CoA and feruloyl-CoA, and the corresponding products were also observed from heptanoyl-CoA, benzoyl-CoA, and phenacetyl-CoA. It is noteworthy that the enzyme synthesized from phenacetyl-CoA an additional product of 200 Da probably corresponding to 5-benzylresorcin. Its formation could be explained by a typical STS-type reaction, i.e. three condensations, STS-type ring folding, and removal of the terminal carboxyl group (see Fig. 1 for the reaction type). Methylation followed by GC–MS analysis revealed a M^+ ion at m/z 228 and a base peak at m/z 91. These results were consistent with the methylation of the two hydroxyl groups in the STS-type product and the appearance of a benzyl fragment.

An STS-type product retaining the terminal carboxyl group (STCS reaction) was not detected from any of the substrates, and none of the identified products or derivatives from them are known from *H. macrophylla*. Therefore it seems most likely that they simply reflected the broad substrate specificity of CHS-related enzymes with non-physiological substrates that has been demonstrated in several instances (Zuurbier et al., 1998; Morita et al., 2000, 2001).

2.4. Activities of STS and CHS with dihydro-4-coumaroyl-CoA

The significance of the stilbenecarboxylate synthesis by the *H. macrophylla* STCS1a from dihydro-4-coumaroyl-CoA was investigated by comparing the activities of purified STS and CHS with that substrate. The STS from *Pinus sylvestris* synthesized the lactones derived from two and three condensations (dihydrobisnoryangonin, dihydro-CTAL). The product from the complete STS reaction (see Fig. 1 for the reaction type) was also identified, as indicated by the appearance of a $[M+H]^+$ ion at m/z 231 in the ESI–MS and the obtained key ions in the CID spectrum: m/z 231 ($[M+H]^+$, 13), 137 (100), 121 (79) and 107 (42). 5-Hydroxylunularic acid was not detected, indicating that the STCS reaction was not carried out by the STS. The physiological substrate cinnamoyl-CoA (apparent K_m 1.5 μM) led predominantly to the stilbene product pinosylvin, but the lactone product after two condensation reactions was also synthesized.

The *P. sylvestris* CHS also accepted dihydro-4-coumaroyl-CoA, and the LC–MS and LC–MS/MS analysis revealed the lactones from two and three condensations as major products. A minor third product could be distinguished by its different elution in the HPLC separation. The analysis by LC–MS/MS (positive and negative ESI-CID, details available on request) showed a fragmentation pattern that could be produced only from the 5-hydroxylunularic acid resulting from a STCS reaction. This appears to be the first example that a stilbenecarboxylic acid may be a byproduct of a CHS reaction, and it is interesting that it was detected with a CHS, but not with the STS. The physiological substrate 4-coumaroyl-CoA (apparent K_m 4.4 μM) led predominantly to naringenin chalcone, but the two lactones resulting from two and three condensations were also detected.

2.5. Malonyl-CoA decarboxylation is strictly coupled to polyketide synthesis in STCS1a, but not in other enzymes of the family

Decarboxylation of malonyl-CoA to an activated acetyl-residue is an integral part of the condensing reaction, and recent work pinpointed the residues in CHS that play the most important roles in this process (Jez and Noel, 2000; Suh et al., 2000a; Jez et al., 2000b). Protonation of the activated form can lead to release of acetyl-CoA, and malonyl-CoA decarboxylase activities have been described for CHS and STS (Jez and Noel, 2000; Suh et al., 2000a,c; Jez et al., 2000b). We became interested in this during the analysis of the CHS-related enzyme pyrone synthase (2PS) from *Gerbera hybrida* that used acetyl-CoA as physiological substrate to synthesize methylpyrone (triacetic acid lactone). In that case the rate of malonyl-CoA decarboxylation to acetyl-CoA was at least five-fold higher than the condensing reactions, and thus it effectively provided the starter CoA-ester from the chain extender (Eckermann et al., 1998). The present investigation aimed at a comparative analysis of four CHS-related enzymes, with particular emphasis on the CHS-related enzyme from *H. macrophylla* because it seemed possible that an STS-type ring folding without loss of the terminal carboxyl group required a particularly tight control of decarboxylase reactions. All of the malonyl-CoA decarboxylase studies were performed in absence and presence of starter substrates in order to see whether the decarboxylase activity was suppressed in the presence of the condensation reactions. The experiments included the use

of the thiol-reactive inhibitor iodoacetamide that had been shown to bind covalently to the active site cysteine and to stimulate malonyl-CoA decarboxylase activity (Jez and Noel, 2000; Suh et al., 2000c). The results for three of the enzymes are summarized in Fig. 6; note the different scales for acetyl-CoA and the products of the condensing reactions.

The pyrone synthase (2PS) was assayed with benzoyl-CoA because it had been shown to be an excellent substrate (Eckermann et al., 1998) and because it permitted the distinction of the products synthesized by the condensing reactions from a non-acetyl-CoA starter (product: phenylpyrone) and from the acetyl-CoA formed by malonyl-CoA decarboxylation (product: methylpyrone).

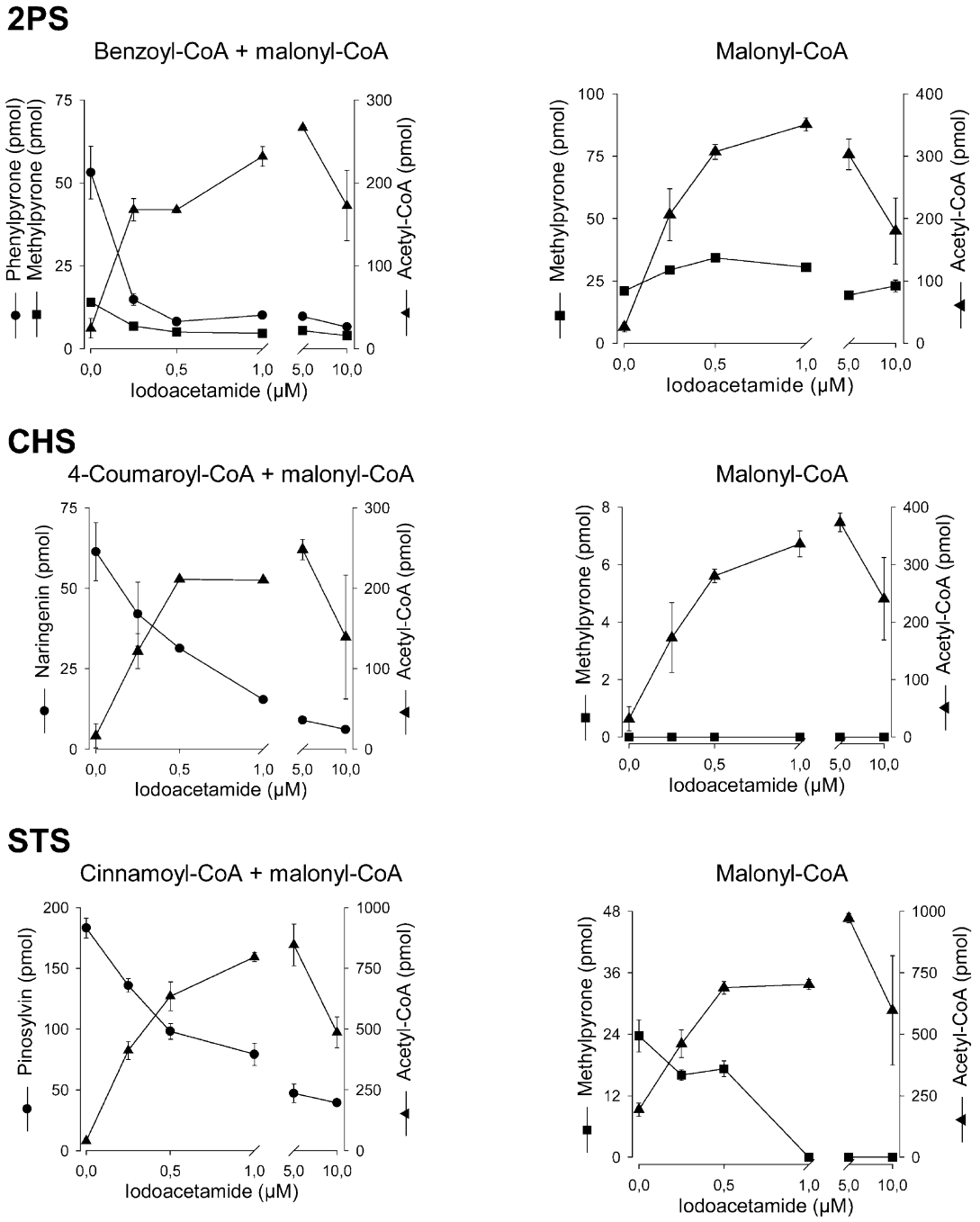

Fig. 6. Malonyl-CoA decarboxylase activity of CHS-related enzymes, and stimulation by iodoacetamide. The incubations were carried out with 15 μM starter substrate (if present) and 14 μM [2-^{14}C]malonyl-CoA. Protein per assay: 2PS, 1 μg; CHS, 1.5 μg; STS, 0.5 μg. The products were quantified after TLC separation. The amounts were calculated from the radioactivity incorporated into the products (based on the number of malonyl-CoA used in their biosynthesis) and normalized to pmol per 1 μg enzyme protein. The bars indicate the extent of variation in the duplicate assays of the results shown in this figure.

The results showed that acetyl-CoA was released in the incubations, and that only part of it was used for the condensing reactions leading to methylpyrone. The condensing reactions with benzoyl-CoA were strongly inhibited by iodoacetamide (I_{50} ca. 0.25 μM), a concentration close to the enzyme concentration in the incubations, suggesting that one or two molecules per enzyme were sufficient to inhibit the condensing reaction. The formation of methylpyrone appeared to be much less affected by the inhibitor, and that was probably a result of the drastic increase in the concentration of acetyl-CoA caused by the dramatic increase of malonyl-CoA decarboxylase activity by iodoacetamide. The results obtained in the presence of only malonyl-CoA indeed suggested that the large excess of acetyl-CoA negated the inhibitory effect of very low concentrations of iodoacetamide.

The results with the CHS and STS from Scots pine (*P. sylvestris*) were comparable in some points, i.e. the release of acetyl-CoA in the standard enzyme incubations, the strong inhibition of the condensing reaction by iodoacetamide, and the dramatic increase of malonyl-CoA decarboxylase activity in the presense of the inhibitor (Fig. 6). However, the assays in the presence of only the chain extender malonyl-CoA revealed an interesting difference between the two enzymes. The acetyl-CoA formed by malonyl-CoA decarboxylation was a good substrate for the STS, but not for the CHS, and this was confirmed by testing acetyl-CoA directly as substrate. The product of the STS reaction was the methylpyrone formed by two condensation reactions, and thus the STS functioned as a 2PS if only malonyl-CoA was present.

These findings showed that all three investigated members of the family, despite their differences in the physiological substrates and reactions, shared the properties that they released acetyl-CoA, that the condensation reactions were inhibited by very low concentrations of iodoacetamide, and that treatment with the inhibitor converted the enzymes into malonyl-CoA decarboxylases. Similar results are known from several other polyketide synthases (Dimroth et al., 1976; Kresze et al., 1977; Shoolingin-Jordan and Campuzano, 1999).

The same experiments were performed with STCS1a. In contrast to all other enzymes of the protein family, it was not possible to detect acetyl-CoA in the incubations, regardless of the starter CoA-ester employed, and it was also not detected in incubations containing only malonyl-CoA. Like with the other enzymes, iodoacetamide was an efficient inhibitor of the condensing reaction, with an I_{50} of ca. 1 μM. On a molar ratio basis this was comparable to the other three enzymes, taking into account that the STCS1a assays contained 5 to 7-fold more enzyme than those for 2PS, CHS, or STS. These enzymes were converted into malonyl-CoA decarboxylases by the inhibitor, but no such effect was observed with STCS1a, and no acetyl-CoA formation was detectable even with iodoacetamide concentrations up to 1 mM. These results revealed a unique property of STCS1a: malonyl-CoA decarboxylation was strictly coupled to the condensation reaction in STCS1a, but not in the other three enzymes.

2.6. Identification of functionally important residues in STCS1a

The functional difference between the CHS-related enzyme from *H. macrophylla* and all other tested members of the family must be based in differences in the amino acid sequence, and therefore STCS1a was inspected for residues that were unusual when compared to other members of the protein family.

One of the striking differences was in position 165, directly adjacent to the active site (Cys164). It is Phe in the vast majority of these proteins, and STCS1a (Fig. 3) was the only example with a His (His165) (except for a predicted CHS from *Humulus lupulus*, GenBank accession AB061021, that otherwise shares only 63% overall identity with STCS1a). This His was potentially interesting because the neighbouring Cys164 and another His (His303) have essential roles in malonyl-CoA decarboxylation (Jez and Noel, 2000; Suh et al., 2000c). His165 was mutagenized to Phe to investigate whether the unusual residue was functionally important. The overall expression of the mutant protein was comparable to that of the parent, but it was almost completely insoluble, and the small amount of soluble recombinant protein that could be purified after large-scale attempts appeared to be complexed with the bacterial chaperone GroEL. The protein revealed no detectable activity with any of the substrates accepted by the parent, and it also had no detectable malonyl-CoA decarboxylase activity. The results indicated that a large hydrophobic residue next to the active site Cys164 apparently blocked the formation of correctly folded enzyme. The His165Phe mutation was then combined with another change suggested by the sequence alignments. In contrast to the vast majority of the other members of the family, STCS1a contained Gly instead of Val in position 170 (Fig. 3). A protein with an additional Gly170Val mutation was constructed, to see whether the second could compensate the first mutation. However, this mutant protein was also insoluble and enzymatically inactive. A change to hydrophobic residues in these residues was apparently not tolerated, and therefore His165 was changed to Asn, a modification that was hoped to improve the solubility and thus the chances to see effects on the enzyme activity. The expression and isolation of the mutant protein confirmed the prediction, although the percentage of soluble enzyme was significantly lower than with the parent protein. The enzyme assays showed

that the mutant had 10–20% of the activity of the parent. No difference in the product profile was detectable, and the mutant also had no malonyl-CoA decarboxylase activity. The data confirmed the importance of His165, but provided no decisive clues on the role in the enzyme reaction.

Finally, we investigated the possible functional importance of the six residue insert LMDSID (Fig. 3) that distinguished STCS1a from all other members of the protein family. Modelling based on the CHS structure suggested that the insertion may be on a surface loop of the protein (not shown). The mutant was less soluble than the parent protein, and the activity was strongly reduced (1–3% of the parent). The product pattern was unchanged, and the mutant had no malonyl-CoA decarboxylase activity.

3. Discussion

CHS-related proteins are key enzymes in the biosynthesis of a large variety of natural products. The plant members presently characterized by DNA sequences and functional assays include proteins performing one, two, or three condensation reactions. The latter group contains at least two types that are distinguished by the type of ring folding to the end product, i.e. the CHSs and the STSs. Several enzymes described by their activities, but not yet by sequence data, are candidates for the family. Almost all of them can be classified to one of these reaction types, and thus probably represent variations of themes already known in principle. One of the rare exceptions are the postulated enzymes synthesizing stilbenecarboxylic acids.

The results with STCS1a from *H. macrophylla* provide the first evidence that proteins from the family of CHS-related enzymes can be stilbenecarboxylate synthases. The enzyme had this activity with only one of the many substrates investigated, dihydro-4-coumaroyl-CoA. This is the precursor for lunularic acid (Fig. 2), a natural product in the Garden Hortensia (*H. macrophylla*), and therefore it is proposed that the enzyme is involved in its biosynthesis. Two other products were also identified (lactones originating from two and three condensation reactions), but this was not unusual or unexpected from in vitro reactions because the corresponding byproducts or derailment products were also detected from reactions of CHSs and STSs with their physiological substrates (Yamaguchi et al., 1999; Suh et al., 2000a,b; Zheng et al., 2001). With all other substrates, including those from the phenylpropanoid pathway, the enzyme produced only the lactone release products after two and three condensations. This is of particular interest for 4-coumaroyl-CoA because it should be the starter for the biosynthesis of hydrangeic acid, the other stilbenecarboxylate in *H. macrophylla*.

Several reasons suggest that additional studies will be necessary to understand the complexities of polyketide biosynthesis in *Hydrangea*, and one is the physiological role of the CHS-related gene. The available results suggest that the genes from *H. macrophylla* and the variety *thunbergii* are functionally identical, but the proposals for the physiological roles are different. Based on the activities with 4-coumaroyl-CoA, the only substrate tested at that time, it was proposed that the enzyme provides a linear tetraketide precursor in the biosynthesis of hydramacroside B (Akiyama et al., 1999). There is no evidence, however, that this substance occurs in the Garden Hortensia, and its structure actually suggests dihydro-4-coumaroyl-CoA as starter substrate (Fig. 4). Our results, on the other hand, indicate that the CHS-related enzyme is a STCS in the biosynthesis of lunularic acid, but it is not likely to have the same role in the variety *thunbergii*, because this compound or derivatives are not known from that plant (Yoshikawa et al., 1994a, 1996a). The two proposals are not necessarily contradictory because both functions could be performed by one enzyme if the roles are solely determined by substrate availability. Nevertheless, it would be interesting to see whether this can be confirmed by other experiments.

Another problem requiring further studies is the STCS activities postulated for the biosynthesis of other stilbenecarboxylates, most importantly hydrangeic acid. This natural product and its derivatives are present in both varieties, and the biosynthesis should start from 4-coumaroyl-CoA. The homology-based PCR techniques had no difficulties with finding the CHS and another sequence sharing only 65% identity with typical CHSs. Dihydro-4-coumaroyl-CoA and 4-coumaroyl-CoA are so similar that it seemed reasonable to think that the STCS for lunularic and hydrangeic acid should be closely related, and that strategies successful for one enzyme should be successful for the other as well. The fact that no additional sequences were detected could simply mean that they escaped attention, a possibility that is difficult to exclude rigorously. We would like to consider another possibility, namely that the identified gene actually has both functions. The idea is attractive because it would explain the failure to find other CHS-related sequences, and STCS1a had the required broad substrate acceptance. The biosynthesis of the different stilbenecarboxylates in the two plants would then be attributed to selective substrate availability (dihydro-4-coumaroyl-CoA and 4-coumaroyl-CoA in the Garden Hortensia; 4-coumaroyl-CoA and caffeoyl-CoA in the variety *thunbergii*).

However, such proposal has to provide an explanation for the finding that 4-coumaroyl-CoA and caffeoyl-CoA led to the derailment products in vitro instead of the stilbenecarboxylates. We would like to argue that this might be a consequence of the fact that the in vitro

conditions lacked an important aspect of the biosynthesis in vivo. The reactions leading to lunularic and hydrangeic acid (Fig. 2) and to the phyllodulcin backbone (Fig. 4) include a reducing step. It is not known at which level it occurs, but there is a precedent in the biosynthesis of reduced chalcones. The formation of 6′-deoxychalcone (Ayabe et al., 1988; Welle and Grisebach, 1988; Hakamatsuka et al., 1988; Welle et al., 1991) involves the co-action of CHS with an enzyme that leads to the reduction of a specific carbonyl group in the tetraketide intermediate, and it was proposed that a reduction at the same position (as shown in Fig. 2) occurs in the biosynthesis of stilbenecarboxylates (Schröder, 1997). Such reducing step is not typical in the biosynthesis of CHS products, but it is remarkable that it must be postulated in the biosynthesis of essentially all stilbenecarboxylates, at least in *Hydrangea* and in liverworts (Gorham, 1995). The correlation suggested that there may be a functional significance, and therefore we looked whether the absence of the reducing step could lead to structural differences in the intermediates that are thought to be the substrates for the ring folding to the end products.

Fig. 7 compares this for dihydro-4-coumaroyl-CoA and 4-coumaroyl-CoA, emphasizing that keto-enol tautomerism permits the formation of conjugated double bonds that might stabilize specific mesomeric resonance states. The lowest number of conjugated double bonds was predicted for lunularic acid formation in vivo, i.e. in presence of the reducing step. There are no data available from *Hydrangea*, but it is noteworthy that the first product identified *in vivo* in the liverwort *Marchantia polymorpha* is prelunularic acid (Ohta et al., 1983, 1984; Abe and Ohta, 1984), a non-aromatic precursor (Fig. 7A). Although other explanations are not excluded, the slow aromatization could be a consequence of the low number of potentially conjugated double bonds in the intermediate. The lack of the reducing step in vitro creates a potential for four conjugated double bonds in the tetraketide part of the intermediate, and the results from the STCS1a indicated that the stilbenecarboxylate was a dominant product, although a significant percentage of the products was dihydro-CTAL that would be considered as derailment product in this scenario. The critical case in this proposal was 4-coumaroyl-CoA because the *Hydrangea* enzyme failed completely to carry out the stilbenecarboxylate ring folding. Fig. 7B shows that the intermediate predicted in vitro (no reducing step) can form a large system of conjugated double bonds that included the aromatic ring of the substrate and thus extended over the entire molecule. The seemingly small difference between dihydro-4-coumaroyl-CoA and 4-coumaroyl-CoA thus can have a major effect on the properties of the intermediates prior to the ring folding. Such extensive mesomeric system is not possible in vivo because it is interrupted as a consequence of the reduction step (Fig. 7B).

The comparison in Fig. 7 suggests a correlation between the potential number of conjugated double bonds in the intermediates and successful STCS-type ring folding. It seems therefore possible that the absence of the reducing step in vitro was one of the main reasons for the failure to obtain stilbenecarboxylates from substrates like 4-coumaroyl-CoA or caffeoyl-CoA. The identification and characterization of the predicted reductases will be necessary to test the proposal.

Several points suggest that stilbenecarboxylate biosynthesis by CHS-related enzymes may require functional specializations that are not common in other members of the family. One may be the presence of a reducing reaction because it seems to be the rule in stilbenecarboxylate biosynthesis, but the exception with CHS, and not known from other proteins of the family. Another unique property of the STCS is the extremely tight control of decarboxylation reactions that was not found with the three other enzymes of the family. It may be possible that the stereo-electronic control to avoid the loss of the terminal carboxyl group concomitant with the STS-type ring folding is a critical part of the STCS reaction. It is tempting to speculate that the unusual His in the position next to the active site Cys participates in this control. The mutagenesis studies showed that it is important for the structure and function of the enzyme, but the mechanisms are not clear, and modelling studies based on the CHS structure failed to provide decisive clues. It is noteworthy, however, that a recent publication reported findings that suggest further experiments with the STCS. It described that a His directly adjacent to the active site Ser of a malonyl-CoA:ACP transacylase could functionally replace the Ser as malonyl acceptor, and the work presented evidence for a malonyl-imidazole adduct (Dreier et al., 2001). It would be interesting to test whether a similar role in malonyl binding is possible with the histidine in the STCS.

4. Experimental

4.1. Plant material, genomic libraries, and PCR primers

H. macrophylla L. plants were obtained from the Botanical Garden of the University Freiburg (Germany). Genomic DNA or RNA were isolated from young leaves. DNA was cloned in the LambdaGEM® Genomic Cloning Vector (Promega GmbH, Mannheim, Germany) as recommended by the manufacturer (Technical Manual #TM029). The following primers were used for the PCR reactions. Sense 1 (surrounding the active site cysteine): 5′-C-A-[T + G]-C-A-[T + G]-G-G-[G + T]-T-G-T-T-T-T-G-C-[T + A]-G-G-[A + T]-G-G-[C + A]-A-C-3′. Sense 2 (downstream of active site cysteine): 5′-G-C-I-A-A-[G + A]-G-A-[T + C]-I-T-I-G-C

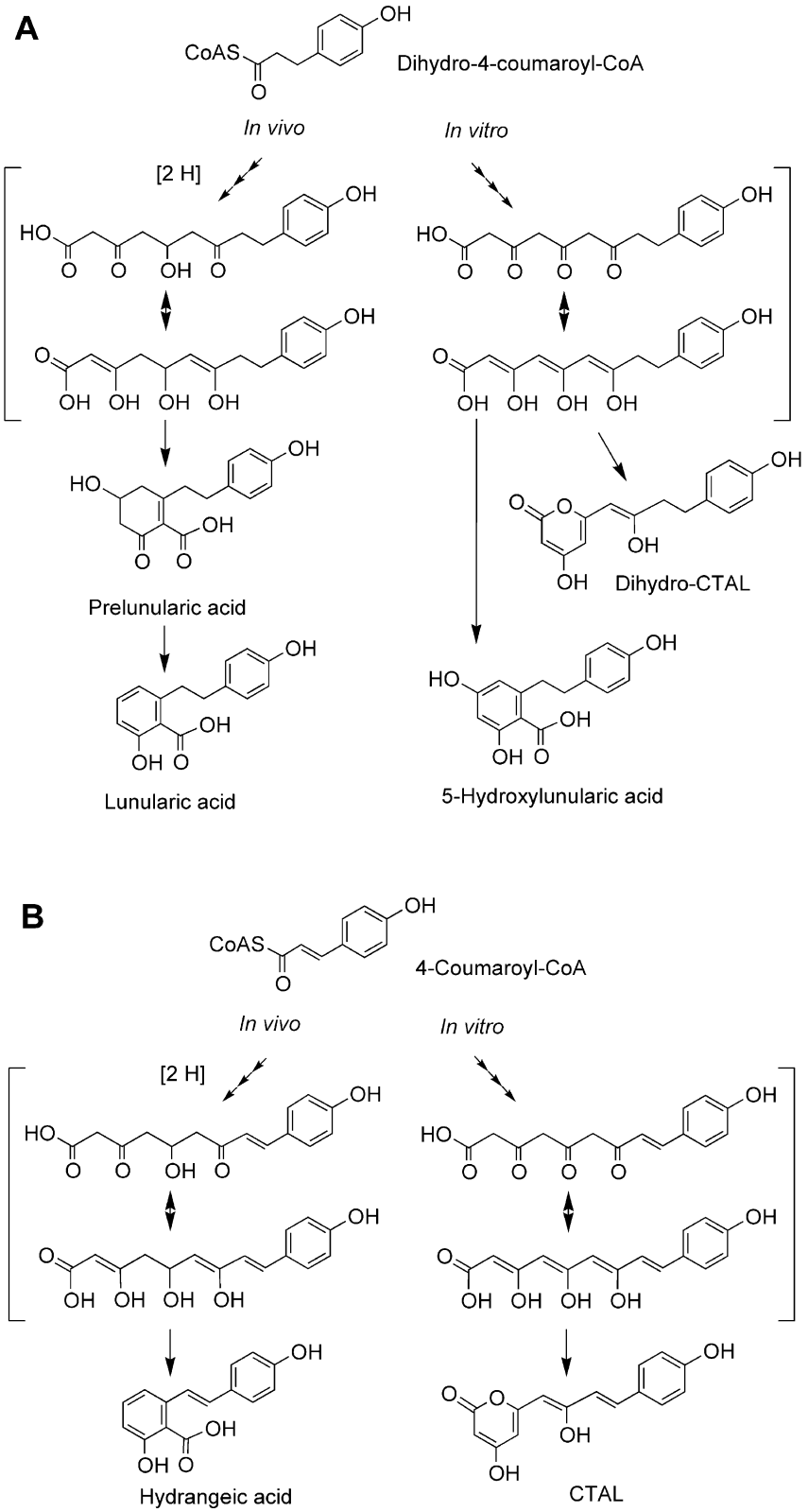

Fig. 7. STCS reactions with different substrates in vivo (left, with reduction step) and in vitro (right, without reduction step). The structures in parentheses describe forms that are possible by keto-enol tautomerism of the linear intermediates resulting from three condensation reactions, prior to the folding of the ring structures. (A) Dihydro-4-coumaroyl-CoA. Prelunularic acid is presumably the initial product of the in vivo biosynthesis, as shown for the liverwort *Marchantia polymorpha*. 5-Hydroxylunularic acid and dihydro-CTAL are found in vitro in the absence of the reducing step. (B) 4-Coumaroyl-CoA. In contrast to all other intermediates, the product from three condensations can form a system of conjugated double bonds that extends over the entire molecule.

-I-G-A-[A + G]-A-A-[C + T]-A-A-[C + T]-A-A-I-G-G-3′. Antisense (conserved FGFG-motif at *C*-terminal): 5′-C-C-[A + T]-G-G-[A + T]-C-C-G-A-A-[G + T]-C-C-G-A-A-[G + T]-A-G-[G + T]-A-C-[T + A]-C-C-C-3′.

4.2. Cloning for protein expression

4.2.1. CHS

The intron in the *HmCHS* gene was deleted by mutagenesis with a 42-bp antisense oligonucleotide joining the end of exon1 and the start of exon 2 (5′-C-T-T-C-T-T-A-A-T-T-T-G-G-G-A-A-T-T-G-T-T-A-C-A-C-A-T-G-C-G-C-T-G-A-A-A-T-T-T-T-G-C-3′). For expression of the protein, a *Bam*HI site was introduced at the *N*-terminal (5′-G-A-A-A-A-T-G-GGATCC-G-T-T-G-A-G-G-A-A-G-T-3′) and a *Hin*dIII site was created a few bp after the stop codon (5′-A-C-AAGCTT-C-C-T-C-T-A-A-G-T-A-G-A-C-A-C-A-C-3′). The *Bam*HI/*Hin*dIII fragment amplified by PCR was then inserted into vector pQE-6 (Crowe and Henco, 1992) for expression of the protein.

4.2.2. CHS-related protein

We used the cDNA (*HmSTCS1a*) for protein expression. A *Nco*I-site was introduced at the 5′-end, directly in front of the start codon (5′-T-G-C-A-CCATGG-C-ATG-G-C-A-A-C-A-A-A-A-A-T-C-G-G-T-A-G-C-A-G-3′), and the 3′-end was modified to contain an *Eco*RI-site directly behind the stop codon (5′-A-G-C-GAATTC-TTA-A-A-T-G-G-G-G-A-C-A-C-T-G-T-G-C-A-A-G-3′). The resulting *Nco*I/*Eco*RI-fragment was inserted into vector pHis8 (Jez et al., 2000b) that provided the fusion to the His-tag for convenient protein purification.

4.3. Expression and purification of proteins

STCS1a (*H. macrophylla*) and the STS from Scots pine (*P. sylvestris* L.) were both cloned for expression in vector pHis8, and the His-tagged proteins were purified according to very similar protocols. *E. coli* BL21(DE) harboring the expression plasmid was grown at 37 °C to an A_{600} of 1.2 in TB medium (Sambrook et al., 1989) with 50 μg/ml of kanamycin. After chilling of the culture on ice, 0.5 mM isopropyl thio-β-D-galactoside (IPTG) was added to induce protein expression, and the culture was incubated further for 4 h at 22 °C. The cells were collected by centrifugation (6000×*g*, 20 min) and washed with 50 mM Tris–HCl buffer (pH 8) containing 0.5 M NaCl, 20 mM imidazole, and 20 mM 2-mercaptoethanol. The cells were disrupted at 1018 psi in a French pressure cell, and the extract was diluted fourfold with the same buffer containing 10 mM 2-mercaptoethanol, 20% glycerol and 1% Tween 20. The suspension was stirred on ice for 30 min and centrifuged (100,000×*g*, 4 °C, 30 min). The supernatant was passed through a Ni-NTA column (Qiagen, Düsseldorf) with Ni^{2+} as affinity ligand. The column was washed extensively with 50 mM Tris–HCl (pH 8) containing 0.5 M NaCl, 10 mM 2-mercaptoethanol, 20 mM imidazole, 20% glycerol and 1% Tween 20, and then with the same buffer without Tween 20. The recombinant proteins were eluted with the same buffer, but containing 250 mM imidazole. The protein solutions were concentrated with Centriprep-30 (Amicon), and the His-tag was removed with 10 U (STCS1a) or 3 U (STS) thrombin protease (Amersham Pharmacia) per mg protein during an 18 h (STCS1a) or 24 h (STS) dialysis at 4 °C against 50 mM Tris–HCl, pH 8, containing 0.5 M NaCl, 20 mM imidazole, 10% glycerol, and 5 mM DTT. The recombinant proteins were further purified by sequentially passing the solutions through the Ni-NTA and a benzamidine Sepharose 6B column (Pharmacia, Freiburg), and by gel filtration (Pharmacia Superdex™ 200 HiLoad) with 25 mM HEPES buffer (pH 7.5) containing 100 mM NaCl and 5 mM DTT. The purification of STCS1a is shown in Fig. 8A, and the purity of the STS is demonstrated in Fig. 8B.

The expression and purification of the CHS from *P. sylvestris* L. and of the 2PS from *G. hybrida* L. have been described (Schröder et al., 1998; Eckermann et al., 1998). The purity of the proteins is shown in Fig. 8B.

4.4. Mutagenesis of HmSTCS1a

The following oligonucleotides were used to change single amino acids (mutated codons underlined). H165->F, 5′-G-ATG-TAC-CAA-CAG-GGG-TGC-TTC-GCG-GGT-GG-3′; H165->N, 5′-G-ATG-TAC-CAA-CAG-GGG-TGC-AAC-GCG-GGT-GG-3′; G170-> V, 5′-GCG-GGT-GGA-ACC-GTC-CTT-CGT-TTG-GCC-3′. The six residue insert (LMDSID) was deleted with the oligonucleotide 5′-GAA-GCT-TTC-ACT-CCA-ATA-|-AGT-ATT-ATT-GAT-TGG-AAC-TCC-ATA-TTC-3′ (-|- = junction).

4.5. Enzyme assays

Standard assays with STCS1a contained 50 mM K-Pi buffer (pH 7.0), 20 μM starter CoA-ester, 28 μM [^{14}C]malonyl-CoA (66,700 dpm), and 6 μg purified protein in a final volume of 50 μl. The reactions were carried out at 30 °C for 15 min and stopped by addition of 5 μl 10% HOAc to obtain pH 4.3. The products were extracted into EtOAc (2×0.2 ml). The incubation times were chosen not to exceed 30% incorporation of the radioactivity. After removal of the solvent in vacuo, the residue was dissolved in 5 μl EtOAc and analyzed on TLC plates (cellulose without fluorescence indicator, or RP18 with fluorescence indicator). The solvents were 20% HOAc and *iso*-PrOH/H_2O/HOAc (60:40:1, v/v/v) for cellulose and RP18-plates, respectively. The incubations for the MS/EIMS analysis contained 30 μM

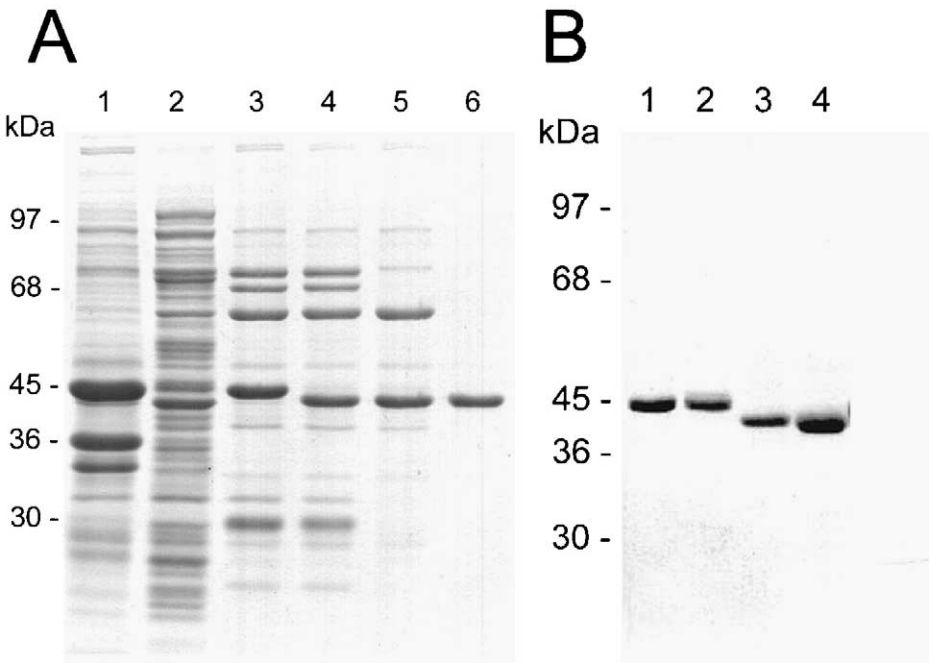

Fig. 8. SDS gelelectrophoretic analysis of recombinant proteins. (A) Purification of the recombinant CHS-related protein from *H. macrophylla* (STCS1a) expressed in *E. coli*. Lanes: 1, pellet obtained after ultracentrifugation of the crude extract (102 μg); 2, soluble proteins after ultracentrifugation of the crude extract (87 μg); 3, after Ni–NTA purification (23 μg); 4, after removal of the His-tag (23 μg); 5, after passage through a combination of Ni–NTA and benzamidine-Sepharose columns (14 μg); 6, after gel filtration (6 μg). (B) Purified recombinant proteins. Lanes: 1, STCS1a (4 μg); 2, CHS from *P. sylvestris* (3 μg); 3, STS from *P. sylvestris* (3 μg); 4, 2PS from *G. hybrida* (5 μg).

unlabelled malonyl-CoA, and the products from 18×0.1 ml incubations with 12 μg enzyme each were pooled. The incubations for the NMR analysis were performed with 50 mM K-Pi buffer (pH 6), 80 μM dihydro-4-coumaroyl-CoA, 120 μM malonyl-CoA, and 170 μg enzyme in a final volume of 1 ml. The extracts from 20 incubations were pooled for the analysis.

The comparisons of STCS1a (*H. macrophylla*), CHS (*P. sylvestris*), STS (*P. sylvestris*), and 2PS (*G. hybrida*) with respect to malonyl-CoA decarboxylase activities were carried out with 0.1 ml incubations containing 0.1 M HEPES buffer (pH 8), 15 μM starter CoA-ester, 14 μM [^{14}C]malonyl-CoA (66,700 dpm), and 1.5–6 μg purified protein (details in text or figure legends). [^{14}C]Malonyl-CoA and [^{14}C]acetyl-CoA were quantified as described (Eckermann et al., 1998). In the experiments with iodoacetamide, the enzymes were preincubated with the inhibitor for 10 min at 30 °C before the reaction start by addition of the substrates.

4.6. Analytical techniques

The product analysis with LC–MS and MS/MS was carried out as described (Eckermann et al., 1998). The methylation of the products was carried out by adding a solution of diazomethane in ether and leaving the mixture overnight at room temperature. The GC/EIMS analysis (Fisons instruments, MD 800) was performed under the following conditions: electron ionisation 70 eV, capillary column DB-5MS (15 m×0.32 mm, film thickness 0.25 μm), injection temperature 250 °C, interface temperature 300 °C, carrier gas helium, flow rate 1.3 ml/min, splitless injection; temperature program: 3 min at 80 °C, increase to 290 °C in 10 min, then hold at 290 °C for 10 min.

The ^{1}H NMR analysis was performed with a Bruker DRX 500 instrument under the following conditions: 500 MHz, 2.5 mm inverse microprobe head, acetone-d_6 for the original sample and MeOH-d_4 for samples obtained after HPLC separation. Trimethylsilane (TMS) served as internal standard. The HPLC separation was carried out with a HP-1100 instrument equipped with diode array detector and a Waters Symmetry C-18 column (5 μm, 4.6×250 mm). The sample in 10 μl methanol was injected manually. The solvent was MeCN/H_2O (30:70 or 10:90, v/v) containing 0.1% trifluoroacetic acid, and the run was for 35 min at 20 °C.

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