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Evidence for Catalytic Cysteine–Histidine Dyad in Chalcone Synthase

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Chalcone and stilbene synthases (CHS and STS) catalyze condensation reactions of *p*-coumaroyl-CoA and three C_2-units from malonyl-CoA, but catalyze different cyclization reactions to produce naringenin chalcone and resveratrol, respectively. Condensing activities of wild-type CHS and STS as well as STS-C60S mutant were inhibited by iodoacetamide (Idm) and diethyl pyrophosphate (DPC). DPC also inhibited malonyl-CoA decarboxylation activity of wild-type and C164S mutants of CHS and STS. Meanwhile, Idm treatment enhanced (two- to fourfold) malonyl decarboxylase activity of wild-type enzymes and STS-C60S, whereas this priming effect was not observed with C164S mutants of CHS and STS, indicating that the cysteine residue being modified by Idm is the catalytic Cys164 of CHS and STS. DPC inhibition of decarboxylation activity of wild-type CHS was pH-independent in the range of pH 5.8 to 7.8; however, its inhibitory effect on CHS-C164S increased as pH increased from 6.2 to 7.4 with a midpoint of 6.4. Based on the 3-D structure of CHS and the observed shift in microscopic pK_a, it was concluded that the histidine residue being modified by DPC in CHS is likely the catalytic His303 and that His303 forms an ionic pair (catalytic dyad) with Cys164 in wild-type CHS. In addition, our results showed that Cys60 in STS is not essential for the activity and only a single cysteine (Cys164) participates in the catalysis as in CHS.

Key Words: **chalcone synthase; stilbene synthase; catalytic dyad; chemical modification.**

Chalcone synthase (CHS, EC 2.3.1.74) and stilbene synthase (STS, EC 2.3.1.95) are multifunctional plant polyketide synthases (PKS) (1). The amino acid sequence identity between these enzymes is higher than 65% and it has been suggested that STSs have evolved from CHSs several times independently (2). Both CHS and STS catalyze a series of reactions up to a common tetraketide intermediate; acyl transfer from a phenylpropanoid starter CoA to a catalytic cysteine (priming) and repetitive decarboxylative Claisen condensations with C_2-units from malonyl-CoA. After each condensation step, CoA-bound ketide intermediate is transferred to the catalytic cysteine for next condensation (Fig. 1). The tetraketide intermediate, either CoA-bound or enzyme-bound, however, is folded differently prior to cyclization to connect different sets of two carbons in two enzymes. In CHS, carbons 6 and 1 are connected by acylation to give chalcone, the precursor for flavonoids and anthocyanin pigments (3). In contrast, in STS, carbons 2 and 7 are brought together for cyclization (accompanied by decarboxylation) to generate stilbene, antifungal phytoalexin (4).

Although CHS and STS catalyze decarboxylative condensations like other condensing enzymes (3-ketoacyl-(acyl carrier protein) synthase, KS) in PKS and fatty acid synthases (FAS), CHS and STS show no significant overall sequence similarity to other condensing enzymes (5, 6). Nonetheless, recent X-ray crystallographic studies revealed that the CHS core structure is similar to those of other condensing enzymes including *E. coli* KS II (Fab F), *E. coli* KS III (Fab H) and *Saccharomyces cerevisiae* thiolase I (7–10). All these proteins display $\alpha\beta\alpha\beta\alpha$ five-layered core structures and are of comparable sizes, homodimers of 34 ~ 45 kDa subunits. Further, there exists striking semblance in the active site residues of these condensing enzymes (see Fig. 4 in Ref. 9). Therefore, proposed catalytic residues in CHS, Cys164, His303, and Asn336 (sequence numbering based on *Pueraria lobata* CHS) correspond to Cys163, His303, and His340 in *E. coli* KS II, and Cys112, His244, and Asn274 in *E. coli* KS III (7–9). All these three catalytic residues are located in the loops converging at the respective active sites.

Abbreviations used: ACP, acyl carrier protein; CHS, chalcone synthase; DPC, diethyl pyrocarbonate; Idm, iodoacetamide; KS, 3-ketoacyl-(acyl carrier protein) synthase; PKS, polyketide synthase; STS, stilbene synthase.

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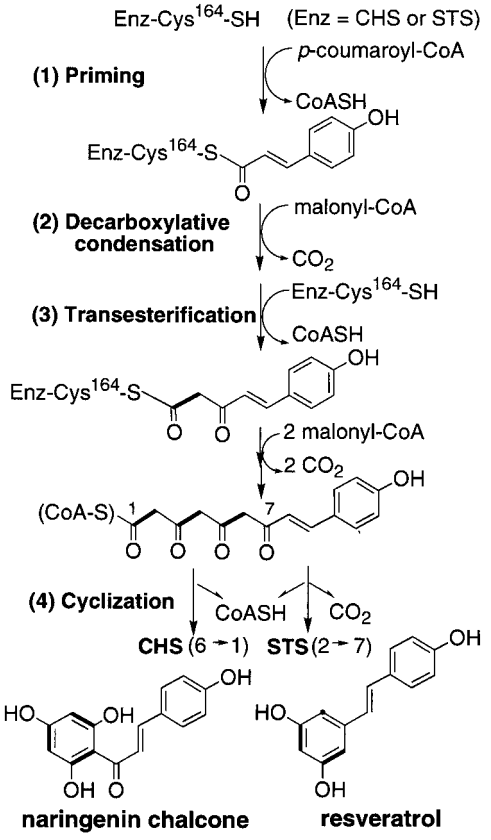

FIG. 1. Reactions catalyzed by CHS and STS. Sequential steps catalyzed by CHS and STS are shown with emphasis on the participation of a single cysteine (Cys164). Naringenin chalcone is chemically converted to naringenin under reaction conditions used.

Earlier, Schröder's group identified Cys164 as the active site nucleophile in CHS, since only the C164A mutant among the mutants of six conserved cysteine residues was completely devoid of activity (11). The crystal structure showed that His303 is located within hydrogen bonding distance of nucleophile Cys164 (7). Further, based on the observation that all the His303 mutants, with a notable exception of H303Q, had no detectable enzyme activity, Jez *et al.* proposed a Cys–His dyad in CHS in that His303 acts as a general base to the thiolate anion of Cys164 (12). Similar catalytic Cys–His dyad was also proposed for KS of actinorhodin PKS (13). In contrast, when His244 was mutated to Ala in *E. coli* KAS III, no effect on transacylation reaction was observed, suggesting that His244, which corresponds to His303 in CHS, has only a minor influence on the nucleophilicity of the catalytic Cys112 (14). Therefore, the existence of a catalytic Cys–His dyad in CHS and in other condensing enzymes is yet to be established.

Meanwhile, there exists a significant difference between CHS (and most likely all other CHS superfamily enzymes) and other condensing enzymes in PKS and FAS. CHS and STS are devoid of a 4′-phosphopantetheinyl moiety and utilize the CoA thioesters of both substrates directly (15). Site-directed mutagenesis study carried out by Schröder and co-workers on CHS and STS showed that there is no second essential cysteine in CHS and STS that accepts the malonyl moiety thus playing the role of the ACP (11). On the other hand, it was suggested that two cysteine residues of grapevine STS, Cys60 and Cys164 are involved in the CoA transfer reactions, thus presenting a major disagreement in reaction mechanism of the CHS superfamily enzymes (16).

Herein, by using combination of chemical modification and site-directed mutagenesis, we present firm evidences that an imidazolium (His303)–thiolate (Cys164) ion pair is indeed present at the CHS active center and that only a single cysteine (Cys164) participates in the STS reaction as in the CHS reaction.

MATERIALS AND METHODS

Site-directed mutagenesis. For improved solubility and easy purification, wild-type and mutant *Arachis hypogaea* STS and *P. lobata* CHS were expressed as thioredoxin-$(His)_6$tag-fusion proteins (Trx-STS and Trx-CHS) in *Escherichia coli* as described previously (17). Site-directed mutagenesis was performed using the PCR megaprimer strategy (18). The forward flanking primers were 5′-GACAAGGCCATGGTGTCTG-3′ (the start codon is in italic and the *Nco*I site underlined) for Trx-STS and 5′-GACAAGGCCATGG-TGAGCGTA-3′ for Trx-CHS. The reverse flanking primers were 5′-TTCGGATCCCCCGTATTATA-3′ for Trx-STS (the *BamH*I site underlined) and 5′-TGTGGATCCAAACTCCAGCAAGT-3′ for Trx-CHS. The mutagenic primers were: For the CHS-C164S mutant, 5′-ACCTGCAAAG**G**AACCTTGTT-3′, for the STS-C60S mutant, 5′-ATCTGTGTTCTCTCA***G****A****T****AT****C***CGC-3′ (*EcoR*V), and for the STS-C164S mutant, 5′-GAA***GG****ATCC*TTGGTGGTACATCAT-3′ (*BamH*I). The codon changed is underlined and mutated bases are in bold. When applicable, a restriction site (in italic) encompassing the mutated codon was created for screening by making additional base changes. All mutagenic primers were antisense.

Following first PCR with the mutagenic primer and the reverse flanking primer, second PCR was carried out with the forward flanking primer and the first PCR products (megaprimer), giving full-length DNA fragment containing the desired mutation. High fidelity *Ex Taq* DNA polymerase (Takara, Japan) was used in the PCR. The mutations were confirmed by DNA sequence analysis using the flanking primers and the dye terminator cycle sequencing kit (Applied Biosystem).

Protein expression and purification. Wild-type and mutant enzymes were expressed in *E. coli* AD494(DE3)pLysS (Novagen) according to the manufacturer's protocol. The LB broth contained 50 μg/ml amphicilin, 34 μg/ml chloramphenicol and 15 μg/ml kanamycin. Induction was carried out at 25°C for 20 h with wild-type enzymes and CHS-C164S mutant and for 10 h with STS mutants. Soluble fraction obtained following the manufacturer's recommendations was applied to a column of Ni^{2+}-iminodiacetic acid Sepharose (Chelating Sepharose Fast Flow, Pharmacia) equilibrated with buffer A (20 mM sodium phosphate buffer, pH 7.4, 300 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM imidazole, 5 mM β-mercaptoethanol). After the column was washed with 80 mM imidazole in buffer A, the fusion proteins were eluted with 250 mM imidazole in buffer A. STS-C60S mutant was further purified by an anion-exchange chromatography. A column of DEAE-ToyoPearl 650M (Tosoh, Japan) equilibrated with buffer B (25 mM Tris/HCl, pH 7.5, 10% glycerol, 0.1% Triton X-100, and 2 mM DTT) was charged with

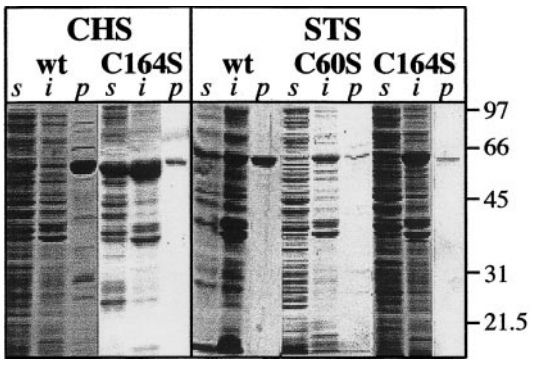

FIG. 2. SDS–polyacrylamide gel electrophoresis of crude soluble (*s*) and insoluble (*i*) *E. coli* extracts showing the expression levels of recombinant proteins and purified (*p*) thioredoxin-fusion wild-type (wt) and C60S and C164S mutants of CHS and STS. Proteins were separated on 12% acrylamide minislab gels and stained with Coomassie brilliant blue R250.

post-Ni^{2+} column solution containing the mutant in buffer B and subsequently washed with a NaCl gradient (50–200 mM). The STS-C60S mutant was eluted at a NaCl concentration of around 165 mM. The purified proteins were stored in buffer C (100 mM potassium phosphate buffer, pH 7.2, 10% glycerol, 0.1% Triton X-100, 1 mM DTT). Protein concentration was determined using Bio-Rad's adaptation of the Bradford dye assay (19) with Trx-CHS as standard.

Chemical modification and enzyme assay. Enzyme (2–6.4 μg) was treated with either iodoacetamide (Idm, final concentration of 0.3 mM) or diethyl pyrocarbonate (DPC, 2 mM) in 40 μl of 0.1 M Hepes, pH 7.5, at 25°C for 40 min in the dark. The reaction with Idm was stopped by addition of DTT (1 mM) and the reaction with DPC was stopped by imidazole (2 mM). For each mutant, control reaction was also run in parallel. To measure the condensation (chalcone or resveratrol producing) activity of the modified enzyme samples, the reaction mixture received 100 μM *p*-coumaroyl-CoA and 17.6 μM [2-^{14}C]malonyl-CoA (Moravek Biochemical, 57 mCi/mmol) and the volume was brought to 100 μl with 0.1 M Hepes buffer (pH 7.5). After incubation at 37°C for 20 min, the reaction products were analyzed by radio-thin layer chromatography as previously described (20). Specific activity was defined as pmol of the product produced/s/mg (pkat/mg).

Malonyl-CoA decarboxylation activity was determined following acetyl-CoA formation (21). At the end of the modification reaction, 35.2 μM [2-^{14}C]malonyl-CoA was added and the Hepes buffer was added to have a final volume of 50 μl. After incubation at 37°C for 20 min, a portion (5–10 μl) was analyzed on silica 60 TLC plate with isopropanol:H_2O:25% ammonia water (80:5:15) as solvent to separate acetyl-CoA (R_f = 0.6) from remaining malonyl-CoA (R_f = 0.25). The specific enzyme activity was expressed in pmol of acetyl-CoA produced/s/mg (pkat/mg).

pH effects on the DPC modification. The reaction mixture contained 3 μg of wild-type CHS or CHS-C164S and 1 mM DPC in 42 μl of 20 mM potassium phosphate buffer of various pH (pH 5.8–8.0). After 40 min at room temperature, the reaction was stopped by addition of imidazole (2 mM) and the pH and buffer concentration were adjusted to 7.4 and 0.1 M by adding appropriate amounts of 1 M K_2HPO_4 and 1 M KH_2PO_4. Then, [2-^{14}C]malonyl-CoA (35.2 μM) was added before incubation at 37°C for 20 min and acetyl-CoA production was quantified as described above.

RESULTS

Expression, purification, and enzyme activity. By virtue of the $(His)_6$-tag located at their N-ends, the wild-type enzymes and the CHS-C164S mutant were obtained in near homogeneity after a single purification step of Ni^{2+}-chelation chromatography (Fig. 2). On the other hand, the majority of the expressed STS-C60S and STS-C164S mutants were recovered in insoluble fractions as inclusion bodies, apparently due to decreased solubility or disturbed folding process. Especially, the recovery of STS-C60S was low and an additional purification step was necessary to achieve comparable purity with other mutants. For this purpose, an ion-exchange chromatography was employed and the mutant was eluted from the DEAE column under essentially identical conditions as used for wild-type STS.

Purified mutants were analyzed for their ability to catalyze the condensing and malonyl-CoA decarboxylation reactions. CHS and STS were shown to catalyze malonyl-CoA decarboxylation and CO_2 exchange both in the presence and absence of *p*-coumaroyl-CoA (15, 16). Decarboxylation of malonyl-CoA initially produces acetyl-CoA carbanion, which is subsequently protonated to acetyl-CoA. In this study, acetyl-CoA formation was followed as a measure of the malonyl-CoA decarboxylation activity in the absence of *p*-coumaroyl-CoA (Fig. 3B). Both CHS-C164S and STS-C164S mutants were devoid of condensing activity and failed to produce even traces of derailment products (20), yet they were active malonyl-CoA decarboxylases (Fig. 3). These results agreed well with earlier study in CHS (11), however, contradicted to the earlier study conducted with grapevine STS, where less than 5% of wild-type activity was observed with STS-C164A mu-

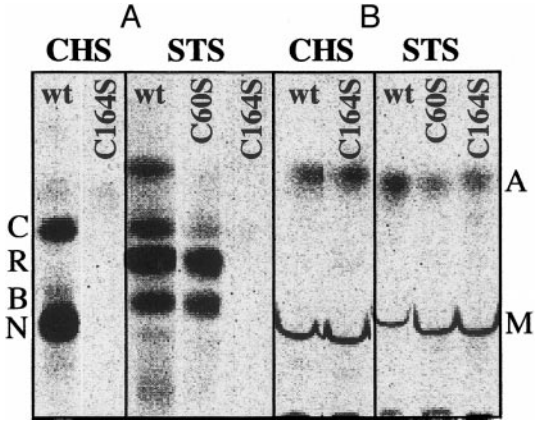

FIG. 3. Radio-thin layer chromatography analyses of the reaction products produced by the wild-type and cysteine mutant CHS and STS enzymes. (A) Condensing activity: Radio-thin layer chromatogram (RP18) of the ethyl acetate extract of the enzyme reaction mixture is shown. The reaction products (N, naringenin; R, resveratrol) as well as derailment products (B, bisnoryangonin; C, *p*-coumaroyltriactic acid lactone) were identified using authentic samples and their known R_f values (20). (B) Malonyl-CoA decarboxylation activity: Radio-thin layer chromatogram (silica 60) of the enzyme reaction mixture is shown. Acetyl-CoA (*a*) and malonyl-CoA (*m*) are identified using their R_f values (21).

TABLE 1

Effects of Iodoacetamide (Idm) and Diethyl Pyrocarbonate (DPC) on Condensation and Malonyl-CoA Decarboxylation Activities

Mutant	Yield[a] (mg/100 ml culture)	Condensation[b,d] Control	Condensation[b,d] Idm (0.3 mM) (pkat/mg)	Condensation[b,d] DPC (2 mM)	Malonyl-CoA decarboxylation[c,d] Control	Malonyl-CoA decarboxylation[c,d] Idm (0.3 mM) (pkat/mg)	Malonyl-CoA decarboxylation[c,d] DPC (2 mM)
CHS-wt	6.9	74 ± 9.5	25 ± 1.5	30 ± 2.2	155 ± 9.2	570 ± 44	54 ± 4.2
CHS-C164S	5.2		Inactive		87 ± 8.2	78 ± 9.1	4.4 ± 0.4
STS-wt	4.6	130 ± 10	11 ± 0.8	8.8 ± 0.5	260 ± 15	650 ± 37	144 ± 8.3
STS-C60S	0.24	1.9 ± 0.23	0.32 ± 0.02	0.21 ± 0.01	69 ± 5.6	94 ± 5.4	28 ± 2.7
STS-C164S	0.63		Inactive		36 ± 4.1	26 ± 3.7	15 ± 3.2

Note. Data are means ± SD (n = 3).

[a] Yields are those of purified enzymes obtained after purification.

[b] Specific activity is defined as picomoles of naringenin (or resveratrol) produced per second per milligram of protein (pkat/mg).

[c] Specific activity is defined as picomoles of acetyl-CoA produced per second per milligram of protein (pkat/mg).

[d] The amounts of enzymes used were CHS-wt and CHS-C164S, 3 μg; STS-wt, 2 μg; and STS-C60S and STS-C164S, 6.4 μg.

tant both in condensing and decarboxylation (16). Both reactions were catalyzed by STS-C60S mutant, albeit at much reduced rates. The condensing activity of this mutant was calculated to be less than 5% of that of wild-type STS and the decarboxylation activity was about 25% of the wildtype. However, the specific activity varied during purification and duration of storage, suggesting that higher amounts of impurity and the mutant's instability contributed to the lower specific activity to some extent.

Chemical modification. As expected, condensing activity of both wild-type CHS and STS as well as STS-C60S were inhibited by thiol-selective iodoacetamide (Idm), presumably due to the labeling of the active site Cys164 in a similar mode to the condensing domain of yeast FAS (22, 23). DPC, a histidine-selective reagent, also inhibited CHS and STS, indicating histidine residue(s) are essential for the activity (Table 1). Towards Idm and DPC, the condensing activity of STS (>90% inhibition) was more sensitive than the condensing activity of CHS (60–65% inhibition) when treated under the identical conditions at pH 7.5. DPC also showed inhibitory effects on decarboxylase activity of wild-type and mutant CHS and STS. Interestingly, DPC more strongly inhibited decarboxylation activity of the C164S mutants than that of wild-type enzymes, and this effect was more prominent with CHS. Thus, DPC at 2 mM inhibited decarboxylation activity of wild-type CHS by 65%, while it inhibited 95% of the activity of CHS-C164S mutant. This enhanced inhibition by DPC indicated that the histidine residue being modified by DPC was better protected in the wild-type enzymes than in the C164S mutants. Presuming the modified histidine is His303 at the active center, one possible explanation would be the existence of imidazolium–thiolate ion pair that reduces the nucleophilicity of His303 in the wild-type but is absent in the C164S mutant. This was further investigated by comparing pH effects on the DPC inhibition of the wild-type and C164S mutant (see below).

In earlier study with yeast FAS, treatment with Idm was found to induce a strong malonyl decarboxylase activity, while abolishing condensing activity (23). Similar inducing (priming) effects were also found with wild-type CHS and STS, but not with the C164S mutants (Table 1), providing a support for that Idm indeed carboxamidomethylates the catalytic Cys164 in CHS and STS. The decarboxylation activity of wild-type CHS and STS and STS-C60S were enhanced after Idm treatment by 3.7-, 2.5-, and 1.4-fold, respectively.

pH effects on the DPC inhibition. To investigate whether there exists an imidazole–thiolate ion pair at the CHS active site, we took advantage of the results that DPC inhibited decarboxylation activity of wild-type and C164S mutant of CHS with different potency at the same pH. Cys164–His303 ion pair should affect not only the microscopic pK_a of Cys164 but also that of His303. These phenomena of perturbed pK_a values for a member of catalytic dyad or triad are well documented in the literature including FAS (22). When the ion pair is destroyed by mutating one of the pair to neutral amino acid, then, the pK_a shift will disappear. The pH/inhibitory activity profile (Fig. 4) showed that inhibition of wild-type CHS by DPC was relatively pH-independent in the range of pH 5.8 to pH 7.8, indicating the pK_a of the histidine residue is not in this pH range. In a sharp contrast, the inhibition of CHS-C164S by DPC increased as pH increased from 6.2 to 7.4 with a midpoint (pK_a) around 6.4, close to the pK_a of 6.5 of free histidine in solution. Taken together with the structural evidence (7), these results were interpreted to suggest that His303 indeed forms an ionic pair with Cys164 in wild-type CHS.

On the other hand, the results with STS were less clear. Inhibition by DPC of the wild-type STS activity began to increase at pH higher than 7.0 (data not

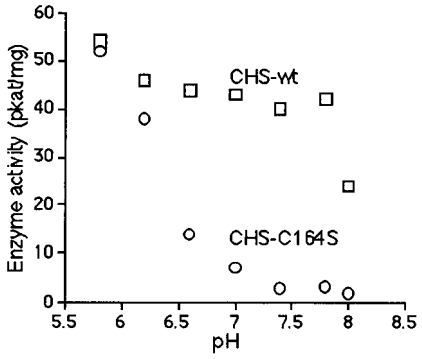

FIG. 4. pH dependence of inhibitory effects of DPC on malonyl-CoA decarboxylation activity of wild-type and C164S mutant of CHS. The reaction mixture contained 3 μg of wild-type CHS (□) or CHS-C164S (○) and 1 mM DPC in 20 mM potassium phosphate buffer of various pH (pH 5.8–8.0).

shown). Due to lower yield and instability of the STS-C164S mutant, we failed to obtain reliable data for the mutant to compare with the wild-type STS. Therefore, it remained undetermined whether the two amino acids (Cys164 and His303) form an ion pair in the STS active center. It seemed possible that the two amino acids in STS, while performing identical catalytic roles, are not as well physically aligned to form an ion pair as in CHS. This may explain the increased sensitivity of STS towards Idm and DPC as compared to CHS under identical conditions.

DISCUSSION

CHS and STS are plant polyketide synthases and represent a group of several closely related enzymes (the CHS superfamily) (1). As a relatively small enzyme (homodimers of 45 kDa subunits), they catalyze multistep reactions including decarboxylative condensations and regiospecific cyclization. Among the many secrets remained to be unveiled about these enzymes, two questions of (i) the existence of catalytic dyad of Cys–His pair at the active site and of (ii) the number of cysteine residues essential for the enzyme activity are studied in this study. First biochemical evidence for the catalytic dyad at the CHS active site was provided by comparing microscopic pK_a values of His303 of the wild-type and C164S mutant by way of studying DPC inhibitory effects on malonyl-CoA decarboxylation. In addition, stimulating effect of alkylation of the active site Cys164 on the decarboxylation was first observed with CHS and STS. This "priming" effect had been observed with FAS and 6-methylsalicylic acid synthase (23, 24). Also, Smith and coworkers recently reported that when active site cysteine of the KAS domain of type I FAS was mutated to glutamine, which is a mimic of Idm treatment, uncoupled malonyl-CoA decarboxylation rate was increased by more than 2 orders of magnitude (25). In a closely related study with PKS, Leadlay's group reported the discovery of KSQ domain in *Streptomyces* aromatic PKS, which contains an conserved glutamine in place of cysteine nucleophile and this KSQ possesses malonyl-ACP decarboxylase activity (26). Taken together, it seems that nature repeatedly employs a same tactic of 'priming' in a variety of related condensing enzymes to prevent premature decarboxylation before the active site nucleophile is charged with a starter CoA.

As shown in Fig. 1, the first step in the CHS and STS reactions is transfer of the phenylpropanoid (e.g. *p*-coumaroyl) moiety onto a catalytic cysteine, which serves the role of the peripheral thiol in FAS (6). There has been a controversy as for the participation of a second cysteine (Cys60) in STS reaction, acting as the central thiol in ACP (11, 16). Our results demonstrated that the C60S mutant of *A. hypogaea* STS not only possesses enzyme activity but also shows virtually identical reactivity towards amino acid selective reagents as compared to the wild-type enzyme. Therefore, it was concluded that Cys60 in STS is not a part of catalytic machinery of STS and decreased activity of the C60S mutant is most likely due to the adverse effects of the mutation on foldability and stability of the STS protein.

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