A Single Change of Histidine to Glutamine Alters the Substrate Preference of a Stilbene Synthase*

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Stilbene and chalcone synthases are related polyketide synthases which use the same substrates but form different products. The environment of the condensing active site cysteine is highly conserved, except for the positions -2 and -3. All chalcone synthases contain Gln-Gln and prefer 4-coumaroyl-CoA as starter CoA ester, while the two known stilbene synthases contain Gln-His or His-Gln (preference phenylpropionyl-CoA and 4-coumaroyl-CoA, respectively). We investigated whether the presence and/or position of the histidine influences the substrate preference and the product specificity (stilbene or chalcone). The two amino acid motifs in the chalcone synthase from Pinus sylvestris (Gln-Gln) and in the stilbene synthases from P. sylvestris (Gln-His) and Arachis hypogaea (His-Gln) were changed by site-directed mutagenesis into all sequence combinations as found in the natural enzymes. Assays changed by site-directed mutagenesis into all sequence motifs in the chalcone synthase from A. hypogaea were different. The change from His-Gln to Gln-His abolished enzyme activity almost completely with all three substrates. The change to Gln-Gln selectively reduced the activity with 4-coumaroyl-CoA, and the kinetic analysis indicated a slight increase in K_m and a 3-fold reduction of V_max, when compared with the parent enzyme. This converted the enzyme from a resveratrol-forming into a dihydropinosylvin-forming stilbene synthase.

Chalcone (CHS) and stilbene (STS) synthases are key enzymes in two different plant-specific pathways (Schröder and Schröder, 1990). Both are polyketide synthases performing a stepwise condensation of a starter CoA ester with three acetate units to a postulated common tetraketide intermediate that is cyclized to ring structures which are different with CHS and STS (Fig. 1). The physiologically most important reaction of CHS is the formation of naringenin chalcone from 4-coumaroyl-CoA, but other starters like cinnamoyl-CoA and phenylpropionyl-CoA are also accepted (Fliegmann et al., 1992). STS is active with the same starters, but the products are stilbenes, and the reaction involves an additional decarboxylation (Fig. 1).

The comparison of CHS proteins from 15 different plants indicates that the enzymes are highly conserved (80% identity) (Schröder and Schröder, 1990). STS have been sequenced from only three different plants (Schröder et al., 1988; Lanz et al., 1990; Melchior and Kindl, 1991; Fliegmann et al., 1992), and the available data suggest that these proteins are more heterogeneous (60-70% identity). Similar values are obtained when the different STS are compared with any CHS, indicating that the two enzyme types are closely related. This presumably indicates that the mechanisms of the condensing reactions are identical or very similar. Recent experiments have shown that the two enzyme types possess a single conserved cysteine (Cys-169), which is essential for the catalytic activity and likely required for the condensing reaction (Lanz et al., 1991).

The environment of Cys-169 is highly conserved in the predicted secondary structure and in the sequences (see Fig. 2 for the examples discussed in this work). A closer inspection, however, indicated some possibly significant differences in the amino acids preceding the cysteine. In the -2 and -3 positions, all CHS possess Gln-Gln without exception, while the known STS contain either His-Gln or Gln-His. STS enzymes are named from the product synthesized with the preferred starter CoA ester, and the three sequenced STS represent two types: (a) the resveratrol-forming STS, which prefer 4-coumaroyl-CoA (from Arachis hypogaea (Lanz et al., 1990; Schröder et al., 1988) and from Vitis var. optima (Melchior and Kindl, 1991)); and (b) the dihydropinosylvin-forming STS from Pinus sylvestris, which prefers phenylpropionyl-CoA (Fliegmann et al., 1992). Interestingly, the two resveratrol synthases contain His-Gln, while the dihydropinosylvin synthase has the sequence Gln-His (Fig. 2).

These differences suggested that the position of the histidine in STS may play a role in the substrate preference and that its presence influences the product specificity of STS and CHS. These possibilities were attractive, because investigations with site-directed mutagenesis showed that a specific histidine may be important for substrate affinity (e.g. adenylate kinase (Reinstein et al., 1990)) or essential for the second part of a complex enzyme reaction (e.g. phosphoenolpyruvate carboxylase (Terada and Izui, 1991)). We tested this with three representative enzymes, the CHS and the STS from P. sylvestris and the STS from A. hypogaea. The two-aminoc acid motifs were changed by site-directed mutagenesis into all three combinations found in CHS and STS proteins. After expression of the mutant proteins in Escherichia coli, we investigated the type of enzyme reaction and the substrate preferences with three different starter CoA esters.
Site-directed Mutagenesis of Stilbene and Chalcone Synthases

3 malonyl-CoA + starter [R] → 3 CoASH + 3 CO₂

Fig. 1. Scheme of reactions performed by CHS and STS. Both perform a stepwise condensation of a starter CoA ester with three acetate units from malonyl-CoA. This is followed by formation of a new ring system which is different in CHS and STS. Large frame, postulated enzyme-bound intermediates. The boxed R indicates that both enzymes are active with several starter CoA esters. Note that the STS reaction involves an additional decarboxylation.

CHS, STS, CHS con...R HMY
predicted structure
CHS con . . . R NH₂
[boxed] C FAGGTVLR

Fig. 2. Environment of the active site cysteine (Cys-169) in CHS and STS. CHS P. sylves., CHS from P. sylvestris (Fliegmann et al., 1992); STS P. sylves., STS (dihydropinosylvin forming) from P. sylvestris (Fliegmann et al., 1992); STS A. hyp., STS (resveratrol forming) from A. hypogaea (Lanz et al., 1990). An extended secondary structure (-----) with a β-turn (>) is predicted for this part of the proteins (program Garnier of the PCGENE software package). Boxed, the active site cysteine (Cys-169) (Lanz et al., 1991) and the two-aminoacid motif investigated in this work. Gin-Gln is strictly conserved in all CHS (CHS con). Double dot, identity; single dot, similarity. The overall relationship between the three proteins is 59% identity and 29% similarity.

EXPERIMENTAL PROCEDURES

Plasmids for Enzyme Expression in E. coli—The constructs for expression of the unmodified enzymes² have been described (Lanz et al., 1991; Fliegmann et al., 1992). All express the complete proteins with transcription under control of a regulated bacterial promoter. The vector plasmids were pTZ19R (Zagursky and Berrin, 1984) for the STS from A. hypogaea and pK253-2 (Amann et al., 1983) for the CHS and the STS from P. sylvestris.

Site-directed Mutagenesis and General Techniques—The mutagenesis was performed with single-stranded DNA produced with helper phage M13KO7 in E. coli strain RJ1332 (Runkel, 1983) as described before (Lanz et al., 1991). The presence of the mutations was verified by DNA sequence analysis with the dideoxynucleotide chain termination technique (Sanger et al., 1977), and the expression of the proteins was confirmed by Western blots as described (Lanz et al., 1991; Fliegmann et al., 1992). Other techniques of molecular biology were carried out according to published procedures (Sambrook et al., 1989).

Computer analysis was performed with the PCGENE software package (IntelliGenetics Inc., Mountain View, CA).

Protein Expression in E. coli, Enzyme Extracts, and Assays—The procedures have been described in detail recently (Fliegmann et al., 1992). Extracts from control E. coli cells do not contain CHS or STS activity (Lanz et al., 1991). The same assay conditions were used with all enzymes and starter CoA esters. Products were analyzed by thin-layer chromatography with 15% acetic acid as solvent. Under these conditions the CHS and STS products are clearly distinguished by their Rf values (Fliegmann et al., 1992). The radioactive products were routinely quantified with a TLC analyzer. The identity of the products was established by HPLC chromatography and gas chromatography-mass spectrometry as described previously (Lanz et al., 1991). The structures of the starter CoA esters and the CHS and STS products are summarized in Fig. 3.

The enzyme activities were not based on total protein, because the rate of protein expression varied among the mutants. Rather, each enzyme extract (soluble proteins after a centrifugation of 10 min at 15,000 × g) was analyzed by Western blotting, and the specific enzyme activity was based on the quantity of cross-reactive material (Lanz et al., 1991).

RESULTS AND DISCUSSION

CHS from P. sylvestris—The enzyme (Gln-Gln) prefers 4-coumaroyl-CoA as compared with cinnamoyl-CoA or phenylpropionyl-CoA (Fig. 4A). The activity with cinnamoyl-CoA is of physiological significance, because P. sylvestris not only contains derivatives of naringenin chalcone but also of pinocebrin chalcone (Sandermann et al., 1989). Changing the motif to an STS type (His-Gln or Gln-His) did not change the product specificity (chalcones) but reduced the activity with all substrates by 40-50% (His-Gln) or more (Gln-His) (Fig. 4A). The results indicated that the presence of two glutamines is not essential for CHS-type enzyme activity, even though the Gin-Gln sequence is strictly conserved in these enzymes. Clearly, introduction of a histidine reduces the overall rate of reaction in a position-dependent manner.

Dihydropinosylvin-forming STS from P. sylvestris—This enzyme has been defined as dihydropinosylvin synthase, because it prefers phenylpropionyl-CoA against the other two

² Accession numbers of DNA sequences: STS P. sylvestris, X60753; CHS P. sylvestris, X60754; STS A. hypogaea, L00952.

Fig. 3. Reaction products of CHS (left) and STS (right) with different starter CoA esters (middle).

A. CHS P. sylvestris
B. STS P. sylvestris
C. STS A. hypogaea

Fig. 4. Activity of the enzymes and the mutants with the starter molecules 4-coumaroyl-CoA (1), cinnamoyl-CoA (2), and phenylpropionyl-CoA (3). All incubations contained ¹⁴C-labeled malonyl-CoA as second substrate. The values are expressed for each series in percent of the activity obtained with the natural protein (sequence flanked by stars) and its best substrate. Experimental variations in the activity in independent assays ±15%.
starter CoA esters (Fliegmann et al., 1992). A change of the original motif Gln-His to the CHS-type Gln-Gln reduced the activity with all substrates (Fig. 4B). The products were the corresponding stilbenes, and no chalcone was detected. The data showed that the presence of a histidine is neither essential for the STS-type reaction nor important for the substrate preference. The low activity of the His-Gln mutant with 4-coumaroyl-CoA was unexpected, because this is the motif present in the resveratrol-forming STS from A. hypogaea that prefers 4-coumaroyl-CoA against the other substrates.

Resveratrol-forming STS from A. hypogaea—The wild-type enzyme (Fliegmann et al., 1992) prefers 4-coumaroyl-CoA, the starter CoA ester leading to resveratrol, the stilbene type found in this plant (Fig. 4C). When compared with the two other enzymes, the mutants of this protein revealed unusual properties. The change from His-Gln to Gln-His led to an almost complete loss of activity with any of the starter CoA esters (Fig. 4C, ≤1% of the original enzyme). The routinely performed Western blots showed that the extracts used for the enzyme activity determinations contained equivalent amounts of immunoreactive protein in the parent enzyme and in the Gln-His mutant. The absence of enzyme activity was therefore not due to instability of the mutant protein. This result, obtained with several independent mutants, was unexpected, because Gln-His is the sequence in the STS from P. sylvestris.

The change from His-Gln to Gln-Gln mutant with 4-coumaroyl-CoA was unexpected, because this is the motif present in the resveratrol-forming STS from A. hypogaea that prefers 4-coumaroyl-CoA against the other substrates. This was unexpected, because Gln-Gln strongly reduced the activity with 4-coumaroyl-CoA. This was unexpected, because Gln-Gln is strictly conserved in CHS, i.e. in enzymes with 4-coumaroyl-CoA as preferred starter CoA ester. When isolated from a plant, all STS with the properties of the Gln-Gln mutant would be classified by standard criteria as dihydropinosylvin-forming STS like the enzyme from P. sylvestris.

The resveratrol-forming STS from A. hypogaea is unusual when compared with the other two enzymes. First, the almost complete loss of activity with the Gln-His mutant indicates that the precise order of the two amino acids is more important with this enzyme than with the STS from P. sylvestris. Second, the change from His-Gln to Gln-Gln altered the substrate preference by a selective reduction of the activity with 4-coumaroyl-CoA. This was unexpected, because Gln-Gln is strictly conserved in CHS, i.e. in enzymes with 4-coumaroyl-CoA as the preferred starter CoA ester. When isolated from a plant, an STS with the properties of the Gln-Gln mutant would be classified by standard criteria as dihydropinosylvin-forming STS like the enzyme from P. sylvestris. The STSs are encoded by gene families in this plant, and several different cloned sequences have been characterized (Fliegmann et al., 1992). However, we have not discovered an exception to the presence of Gln-His in STSs and Gln-Gln in CHS. Dihydropinosylvin has been described as a common constituent in Pinus species (Gorham, 1989), and it would be interesting to determine whether other members of this plant family show the same conservation of the two-aminoacid motif in STS and CHS.

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REFERENCES

Amann, E., Brosius, J., and Ptashne, M. (1983) Gene (Amst.) 25, 167-178
Fliegmann, J., Schroeder, G., Schantz, S., Britsch, L., and Schroeder, J. (1992) Plant Mol. Biol. 18, 488-503
Gorham, J. (1989) in Methods in Plant Biochemistry (Dey, P. M., and Harborne, J. B., eds) pp. 169-196, Academic Press, London
Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 496-492
Lanz, T., Schroeder, G., and Schroeder, J. (1990) Planta (Heidelberg) 181, 169-175
Langer, J., Tropf, S., Marner, F.-J., Schroeder, J., and Schroeder, G. (1991) J. Biol. Chem. 266, 9871-9876
Melchior, F., and Kindl, R. (1991) Arch. Biochem. Biophys. 288, 552-567
Reinsteins, J., Vetter, I. R., Schlichting, I., Reusch, P., Wittig, and Hauser, A., and Roos, G. S. (1990) Biochemistry 29, 7420-7450
Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sandermann, H., Jr., Schmitt, R., Hell, W., Rosenstem, D., and Langebail, C. (1989) in Acid Deposition: Sources, Effects, and Controls (Longhurst, J. W., S., ed) pp. 243-294, British Library, London
Sanger, J., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467
Schroeder, G., and Schroeder, G. (1990) Z. Naturforsch. Sect. C Bioc. 45, 1-8
Schröder, G., Brown, J. W. S., and Schroeder, J. (1988) Eur. J. Biochem. 172, 161-169
Terada, K., and Imai, K. (1991) Eur. J. Biochem. 202, 797-803
Zagursky, R. J., and Berman, M. L. (1984) Gene (Amst.) 27, 183-191

3 S. Schantz and G. Schröder, unpublished results.