Identifying functional domains within terpene cyclases using a domain-swapping strategy

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Communicated by Bernard O. Phinney, University of California, Los Angeles, CA, March 18, 1996 (received for review September 20, 1995)

ABSTRACT Cyclic terpenes and terpenoids are found throughout nature. They comprise an especially important class of compounds from plants that mediate plant–environment interactions, and they serve as pharmaceutical agents with antimicrobial and anti-tumor activities. Molecular comparisons of several terpene cyclases, the key enzymes responsible for the multistep cyclization of C_{10}, C_{15}, and C_{20} allylic diphosphate substrates, have revealed a striking level of sequence similarity and conservation of exon position and size within the genes. Functional domains responsible for a terminal enzymatic step were identified by swapping regions approximating exons between a Nicotiana tabacum 5-epi-aristolochene synthase (TEAS) gene and a Hyoscyamus muticus vetispiradiene synthase (HVS) gene and by characterization of the resulting chimeric enzymes expressed in bacteria. While exon 4 of the TEAS gene conferred specificity for the predominant reaction products of the tobacco enzyme, exon 6 of the HVS gene conferred specificity for the predominant reaction product(s) of the Hyoscyamus enzyme. Combining these two functional domains of the TEAS and HVS genes resulted in a novel enzyme capable of synthesizing reaction products reflective of both parent enzymes. The relative ratio of the TEAS and HVS reaction products was also influenced by the source of exon 5 present in the new chimeric enzymes. The association of catalytic activities with conserved but separate exonic domains suggests a general means for generating additional novel terpene cyclases.

Terpenes and terpenoids constitute a very large family of compounds. Approximately 20,000 different terpenes and terpenoids have been identified to date, representing only a small fraction of the estimated natural variation. The terpenoid structures are diverse and range from relatively simple linear hydrocarbon chains to highly complex ring structures. Cyclic terpenoids include monoterpenes (10 carbons) derived from geranyl diphosphate, sesquiterpenes (15 carbons) and sterols (30 carbons) derived from farnesyl diphosphate, and diterpenes (20 carbons) derived from geranylgeranyl diphosphate. These compounds are found throughout nature but comprise an especially important class of compounds in plants; compounds that mediate plant–plant, plant–insect, and plant–pathogen interactions (1–3). From a health-related perspective, these compounds represent important classes of antimicrobial and chemotherapeutic agents (4–7).

The biosynthesis of cyclic terpenes is determined by key branch point enzymes referred to as terpene cyclases, or more properly, terpene synthases. The reactions catalyzed by terpene cyclases are complex, intramolecular cyclizations that may involve several partial reactions (8, 9). For example, the bioorganic rationale for the cyclization of farnesyl diphosphate by two sesquiterpene cyclases pertinent to this work are illustrated in Fig. 1A. In step 1, the initial ionization of farnesyl diphosphate is followed by an intramolecular electrophilic attack between the carbon bearing the diphosphate moiety and the distal double bond to form germacrene A, a macrocyclic intermediate. Internal ring closure and formation of the eudesmane carbonon ion constitutes step 2. For tobacco 5-epi-aristolochene synthase (TEAS), the terminal step is a hydride shift, methyl migration, and deprotonation at C9, giving rise to 5-epi-aristolochene (step 3a). Hyoscyamus muticus vetispiradiene synthase (HVS) shares common mechanisms at steps 1 and 2, but differs from TEAS in the third partial reaction, in which a ring contraction would occur due to alternative migration of an electron pair. In each case, a monomeric protein of ~64 kDa catalyzes the complete set of partial reactions and requires no cofactors other than Mg^{2+}.

Numerous terpene cyclases from plant and microbial sources have been partially or completely purified and characterized (11–22). These enzymes behave as soluble enzymes with molecular weights of 40,000–100,000. Mechanistic studies have included evaluations of the proposed reaction mechanisms (23–27), efficacy of substrate analogs (28, 29) and suicide inhibitors (28–30), and the use of chemical-modifying reagents and site-directed mutagenesis to identify amino acids essential for catalysis (31–33). Recently, a number of fungal and plant genes for monoterpenes, sesquiterpenes, and diterpene cyclases have been described (34–41). The plant monoterpene, sesquiterpene, and diterpene cyclases exhibit a significant degree of similarity at the amino acid level, and at least for the sesquiterpene and diterpene cyclases, the intron/exon organization of genomic DNA is nearly identical (40–42). In contrast, other than perhaps the conservation of 5-sequences rich in aspartate residues, very little similarity is observed between the fungal and plant sesquiterpene cyclase proteins (38). One implication of the sequence similarity and conserved intron/exon organization observed between the plant genes is that regions of sequence conservation may correspond to functional domains, and those functional domains may mediate the catalysis of partial reactions. This inference was recently tested by comparing the deduced amino acid sequence of the TEAS and HVS sesquiterpene cyclases (77% amino acid identity; ref. 41). Although chemical rationalization of the reaction mechanisms strongly suggests several partial reactions common to both enzymes and at least one final partial reaction unique to each (Fig. 1A), there is a more or less equal distribution of amino acid substitutions and mismatches throughout the deduced amino acid sequence comparisons. This level of conservation and equal dispersal of amino acid substitutions did not allow us to readily identify domains that might contribute to common, partial reactions between the two enzymes, nor those regions that might contribute to the unique terminal steps. Therefore, a functional assay employing a domain-swapping strategy was developed to map those regions of the cyclase protein contributing to...
FIG. 1. (A) Proposed reaction mechanisms for eremophilane (TEAS)- and vetispiradiene (HVS)-type sesquiterpene cyclases. Partial reactions 1 and 2 are considered common to both types of cyclases. Mechanistic differences in partial reactions 3a and 3b are sufficient to account for the different reaction products shown. Adapted from refs. 9 and 10. (B) Schematic diagram of a domain-swapping test. Expression of the indicated genes in bacteria is expected to generate sesquiterpene cyclases that catalyze reactions, resulting in one of two possible reaction products.

to the last partial reaction. Based on the reaction mechanisms proposed in Fig. 1A, we predicted that a single amino acid domain could be sufficient to account for the last partial reaction catalyzed by the tobacco and Hyoscyamus enzymes and that this domain would be found in analogous positions within the proteins (Fig. 1B).

MATERIALS AND METHODS

Construction of Chimeric Genes. Full-length cDNAs for TEAS and HVS were initially cloned into the EcoRI-XhoI sites of pBluescript SK (Stratagene), creating pBSK-TEAS and pBSK-HVS (41). Each clone consisted of the translation start codon neighboring the EcoRI restriction site and the 3'-poly(A) tail of the cDNAs flanked by the XhoI restriction site. Chimeric constructs (CH1, CH2, CH5, and CH7) were constructed on the basis of conserved HindIII and NdeI restriction sites between the two genes. For example, CH1 (pGBT-CH1) was prepared by ligating the 5'-terminal portion of the TEAS gene (corresponding to the EcoRI–HindIII fragment) with the 3'-terminal portion of the HVS gene (corresponding to the HindIII–KpnI fragment) into the bacterial expression vector pGBT-T19 (Gold Biotechnology, St. Louis) predestined with EcoRI and KpnI. Ligation products were transformed into E. coli strain TB1 according to a standard CaCl2 transformation procedures. CH2, CH3, and CH7 were constructed in a similar manner. CH3, CH4, CH12, and CH13 were constructed using a PCR strategy wherein primers containing convenient restriction sites were designed for the amplification of particular segments of the HVS gene. For example, CH3 was constructed as follows. An EcoRI–ClaI restriction fragment of the TEAS

gene was isolated and ligated together with a ClaI–KpnI isolated insert of the HVS gene [prepared by standard PCR methodology using 5'-d(GGGATCGATGACATAGCCAGTATGAGGTT)-3'] as the forward primer and 5'-d(AATACGACTCACTATAG)-3' as the reverse primer (this primer corresponds to the T7 sequence found in the multiple cloning site of pBSK) using pBSK-HVS as the template] into the EcoRI–KpnI sites of the pGBT-T19 vector. CH4 and CH13 were constructed in a similar manner except with the forward primer 5'-d(CGAGTCAACTATAGGTTATTGAGGATA)-3' (HinII restriction site underlined) for CH4 and the forward primer 5'-d(TATCTCATGGGATTATGAA)-3' (XbaI restriction site underlined) for CH13. CH12 was prepared by ligating a PCR fragment corresponding to the first 1326 nt of CH4 [forward primer 5'-d(GGGATGTCAATTCATTGGCCTACAGCAGTGCAAACAT)-3' (EcoRI restriction site underlined) and translation codon in boldface type] and the reverse primer 5'-d(GGAATCGATGACATAGCCAGTATGAGGTT)-3' (ClaI restriction site underlined)] with the ClaI–KpnI fragment of the TEAS gene into the EcoRI–KpnI sites of the pGBT-T19 vector. Ligation of the EcoRI–HindIII fragment of the HVS gene with the HindIII–KpnI fragment of CH3 generated CH6. CH8 was created by ligating the EcoRI–NdeI fragment of HVS with the NdeI–KpnI fragment of CH3. CH9 was created by ligating the EcoRI–NdeI fragment of CH5 with the NdeI–KpnI fragment of CHS. CH10 was created by ligating the EcoRI–HindIII fragment of HVS with the HindIII–KpnI fragment of CH4. CH11 was created by ligating the EcoRI–NdeI fragment of HVS with the NdeI–KpnI fragment of CH4. CH14 was created by substituting the EcoRI–NdeI fragment of pGBT-CH13 with the corresponding DNA fragment of pBSK-HVS. The junctions of the CHs were confirmed by double-stranded DNA sequencing using the dideoxy nucleotide chain termination method according to the manufacturer (United States Biochemical).

Bacterial Expression Studies. Escherichia coli TB1 cells were used for all the expression studies. Procedures for growth of the bacterial cells, induction of gene expression, and measurement of sesquiterpene cyclase enzyme activity and total protein in the bacterial lysates were identical with those described (41, 43). The predominant reaction products were separated from one another by developing G60 silica TLC plates impregnated with 15% silver nitrate in benzene:hexane:diethyl ether (50:50:1). For qualitative evaluations, the TLC plates were sprayed with En3hance surface fluorography spray (DuPont) and exposed to Kodak XAR-5 film for 2–5 days at −70°C. For quantitative evaluations, 0.5-mm zones of an entire lane were scraped into scintillation vials, and the radioactivity determined in a Packard 1500 liquid scintillation counter. The dominant reaction products generated by the cyclase activities resulting from expression of the TEAS, HVS, CH4, and CH14 constructs in bacteria were also verified by GC and GC-MS using equipment and conditions described (ref. 44 and data not shown). The mass spectra profiles were compared with that published for 5-epi-aristolochene (46), and the predicted fragmentation pattern for vetispiradiene from the work of Enzell et al. (45).

RESULTS

Product-Specificity Domains. CHs were generated by substituting the corresponding domains between the tobacco and Hyoscyamus genes, the chimeric genes were expressed in bacteria, and bacterial lysates were assayed for sesquiterpene cyclase activity (43). The sesquiterpene cyclase assays included an argentation-TLC analysis that easily distinguishes the TEAS-specific and HVS-specific products from one another (41). Fourteen CHs were generated and assayed in this manner, and the results from this analysis are presented in Fig. 2.
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Comparison of CH2 and CH3 provides evidence for specificity of end-product formation residing within a domain of ~181 aa, corresponding to the NdeI and ClaI restriction sites within the genes. CH4, however, presents an unexpected result. Expression of CH4 results in a cyclase protein capable of generating reaction products reflective of both the TEAS and HVS enzymes. One interpretation of this result is that amino acids 261–379 within the tobacco protein are responsible for the TEAS-specific products (corresponding to the NdeI to HincII fragment of the cDNA), whereas amino acids 379–442 within the Hyoscyamus protein are responsible for the HVS-specific products (corresponding to the HincII to ClaI fragment of the cDNA). This interpretation is confirmed in constructs CH11 and CH12. CH11 represents the substitution of the NdeI to HincII fragment of the Hyoscyamus gene with the corresponding tobacco gene fragment and results in a multifunctional enzyme. CH12 represents a substitution of the HincII to ClaI fragment of the tobacco gene with the corresponding Hyoscyamus gene fragment and results in a multifunctional enzyme. Comparing CH11 with CH13 provides a further refinement in mapping that domain of the tobacco enzyme responsible for the TEAS-specific products. Because CH13 is a multifunctional enzyme, this construct suggests that the 81 aa encoded by the DNA fragment residing between the NdeI to XbaI restriction sites of the tobacco cDNA are sufficient for formation of the predominant TEAS-specific products. This interpretation is confirmed by substituting only this domain within the HVS cDNA with that of the TEAS gene (CH14; Fig. 2A).

The predominant reaction product(s) of the wild-type tobacco and Hyoscyamus genes expressed in bacteria migrated on silver nitrate-TLC plates with Rf values of 0.41 and 0.31 (Fig. 2B), values consistent with our previous characterization of these products as 5-epi-aristolochene and vetispiradiene, respectively (41, 43). Additional analysis by GC and GC-MS indicated that the predominant TEAS reaction products were 5-epi-aristolochene (70% of total products, based on percentage of total peak areas from GC analysis; ref. 46) and another bicyclic sesquipiperone (20%; [M]+ ion at m/z 204) whose exact structure is currently unknown, the dominant HVS reaction product was vetispiradiene (> 90%) ([M]+ ion at m/z 204 with a base peak at m/z 41 and a series of predictable ions at m/z 175, 108, 94, and 68; refs. 43, 45, and 47), and the predominant reaction products of CH4 were 5-epi-aristolochene (18%), the bicyclic sesquipiperone of unknown structure (43%), and vetispiradiene (32%; data not shown). Other studies relying on affinity purification of histidine-tagged recombinant cyclase proteins has revealed five other minor reaction products, each representing ~1% of the total products, with all five found at the same relative abundance in all the reaction assays. The detailed structures of these minor sesquipiperone products are not known at this time.

**Ratio-Determinant Domain.** Another important domain of the cyclase proteins was identified by comparing the relative ratios of the predominant reaction products produced by the multifunctional enzymes (Fig. 2). For example, the reaction products resulting from expression of constructs CH4, CH10, shown) in bacterial lysates of CH7, yet no enzyme activity was detected. The lack of positive results from the CH7 construct does not negate those of CH1 and CH2, but suggests that there must be interactions between the carboxy and amino terminal portions of the protein that are accommodated in some constructs and not in others. This is further emphasized by comparing the specific activity of the enzymes generated by CH5 and CH6. CH5 results in a 10-fold lower specific activity of cyclase enzyme activity even though the absolute level of expressed protein is similar to the other constructs (determined by immunodetection; data not shown). Substituting a HVS carboxy terminal region restored the specific activity to the cyclase enzyme generated by CH6.
CH11, and CH12 were generated in a ratio of 60–70% TEAS-specific to 30–40% HVS-specific. In contrast, the ratio of reaction products resulting from expression of constructs CH13 and CH14 was essentially the inverse. This result implies that the region encompassed by the XbaI to HincII domain influences the relative ratio of reaction products generated by the multifunctional enzymes.

**DISCUSSION**

Based on chemical rationalizations for the reactions catalyzed by the tobacco 5-epi-aristolochene synthase and the *H. muticus* vettispiradiene synthase (9, 10), we predicted that a single amino acid domain could be sufficient to account for the last partial reaction catalyzed by these enzymes and that this domain could be found in analogous positions within the proteins (Fig. 1 A and B). This was then tested by complementary substitution of cdNA regions approximating exons between the cdNA clones, expression of these chimeric genes in bacteria, and biochemical characterization of the bacterial expressed sesquiterpene cyclase enzyme and reaction products. The unexpected finding of a chimeric sesquiterpene cyclase activity that generated a reaction product profile characteristic of both parent cyclase enzymes is not consistent with this initial hypothesis. One possible explanation for this result is based on the observation that many terpene cyclases are known to generate multiple reaction products (15, 21, 22, 26) and assumes that both the wild-type TEAS and HVS cyclase possess low-level or latent activity for additional reaction products. Substituting the Ndel–Xbal domain of wild-type HVS with that of TEAS (CH14 in Fig. 2 A), for example, would then be sufficient to create enough of a structural change in the chimeric protein to allow for expression of a latent or low-level activity endogenous to other domains of the HVS protein. This possibility requires, however, that the different domain swaps (the Ndel–Xbal domain of HVS for that of TEAS and the HincII–Clal domain of TEAS for that of HVS) uncover different latent or low-level activities in the different CHs. An alternative interpretation is shown in Fig. 3, wherein two separate and distinct domains within the cyclase peptide contribute directly to the type of end-products generated and are interrupted by another domain which we refer to as the ratio-determinant domain because of its influence on the distribution of end-products. This alternative model also makes assumptions, the most important of which is that the chimeric genes code for one protein with multiple activities and that the initial translation products cannot fold into two possible conformations, each conformer being capable of a different reaction product profile.

The model in Fig. 3 poses several intriguing questions. For example, how position-dependent are these domains? Can the HVS-specific domain be translocated to that region of the protein containing the TEAS domain and remain active? Is the reciprocal construct active? Other questions relate to how the multifunctional activity of the enzyme must come about. Assuming that the chemical reactions up to the germacrene A intermediate are common within the protein structures, then the germacrene intermediate must vibrate, rotate, or twist within the reaction pocket sufficiently to expose particular bonds and atoms to particular reactive amino acid residues/domains driving one or the other final step. Exactly how to test these possibilities is somewhat more problematic, but will likely require the synthesis of alternative intermediates that can tag amino acid residues involved in various steps of catalysis (30) and additional structural mutants that perturb physical changes in the enzyme molecule undergoing catalysis.

The current results add an important functional consideration to the conserved gene structure observed within plant terpene cyclases (40–42). Alignment of amino acid sequences corresponding to exons for the mint monoterpene cyclase, the tobacco, *Hyoscyamus*, and cotton sesquiterpene cyclases and the castor bean diterpene cyclase exhibit an exceptional degree of sequence similarity (>50% similarity overall). All five enzymes share mechanistic similarities to one another but also must contain catalytic activities directing substrate and product specificity. The current results demonstrate a close correspondence between exons 4, 5, and 6 and discrete functional activities. Whereas ascribing functional attributes to exons 1–3 and 7 must await further analysis, the current work clearly predicts that substitution of exons 4 and 6 of CH4, for example, with those corresponding to the mint monoterpene or castor bean diterpene cyclases could create new terpene cyclases capable of synthesizing novel reaction products.

We thank Dr. Charles West for stimulating discussions; Drs. Joel Noel, Tom Pirone, Skip Waechter, and David Watt for their critical assessment of this work; and Dr. Harold Burton and Mr. Ziaochen Wei for their assistance with the GC–MS analyses. This work was supported by a grant from the National Science Foundation. This is journal paper 96-06-093 from the Kentucky Agricultural Experiment Station.

Biochemistry: Back and Chappell