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Mutational analysis and dynamic simulation of S-limonene synthase reveal the importance of Y573: Insight into the cyclization mechanism in monoterpene synthases

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ABSTRACT

Monoterpene synthases carry out complex reactions to produce multiple products from a sole substrate, geranyl pyrophosphate (GPP). S-limonene synthase (LS) is a model monoterpene synthase that can be explored to understand the catalytic mechanism of these enzymes. In this study, we have identified an active site tyrosine residue (Y573) is crucial for the enzyme activity and mutational analysis indicates that both the aromatic ring and hydroxyl group are essential for the catalysis. Dynamic simulations found a hydrogen bond between Y573 and D496 and also a significant conformational change in the helical form of the LPP intermediate. Further mutagenesis suggested that this hydrogen bond is essential for catalysis. Sequence analysis suggested Y573 is completely conserved among cyclic monoterpene synthases but variable in acyclic enzymes, indicating this residue may be involved in cyclization. Subsequent studies by using neryl diphosphate (NPP) as the substrate ruled out the possibility that Y573 functions solely at the substrate isomerization step. Therefore, a more complicated role may be played by this residue. We proposed that Y573 may be involved in the earlier steps of the reaction, probably by controlling the conformation of the helical LPP intermediate. Our study provides important insights not only on the catalytic mechanism of LS, but also on the cyclization of monoterpene synthases in general.

1. Introduction

Terpenes comprise a structurally diverse group of metabolites and play critical roles in plants by defensing against pests and pathogens [1–3]. It has been estimated that there are more than 60,000 different terpene metabolites [4]. The diversity of terpenes is attributed to terpene synthases [5–8]. Terpene synthases carry out complex reactions and transform the prenyl diphosphates, into skeletal structural diverse terpenoids. Many terpene synthases possess promiscuous activities to produce multiple products. For example, γ-humulene synthase from *Abies grandis* generates 52 kinds of sesquiterpenes from a sole substrate, farnesyl pyrophosphate (FPP) [9–11].

The structural complexity of terpene synthases underlies the structural diversity of products. Despite their diverse biological activities, terpene synthases share a common active sites scaffold [12–14]. They are termed into two classes based on their structural characters. The two distinct classes have different reaction cascades. In the class I terpene synthases, it is initiated by elimination of the allylic diphosphate from the prenyl diphosphate substrate to yield a carbocation intermediate. Subsequently, the carbocation intermediate undergoes a series of reactions, including isomerization, cyclization, hydride shift, and other rearrangements. Finally, the reaction is terminated by deprotonation or the addition of a nucleophile, and a large number of terpenes with different regio- and stereochemistries are produced.

Of especial interest is how these enzymes specify the product profile. It can be affected by many factors, because of the complicated reaction mechanisms. Stabilization, rearrangement and quenching of the carbocation intermediate have significant influences on the product profile during the cyclization [11–13]. Recent reports have demonstrated that products can be dramatically altered by changing a small number of amino acid residues [11,15–17]. Our previous study on 4S-limonene synthase (LS) from *M. spicata* indicates that the polarity of N345 is critical for the formation of limonene, because of it stabilizes the (4S)-α-terpinyl cation [17]. Srividya and colleagues also suggest residues W324 and H579 in LS are involved in the stabilization of the (4S)-α-terpinyl cation by the interaction between the carbocation and the π-system of the aromatic side chain [18]. Both residues may also participate in the final deprotonation step. In addition, the

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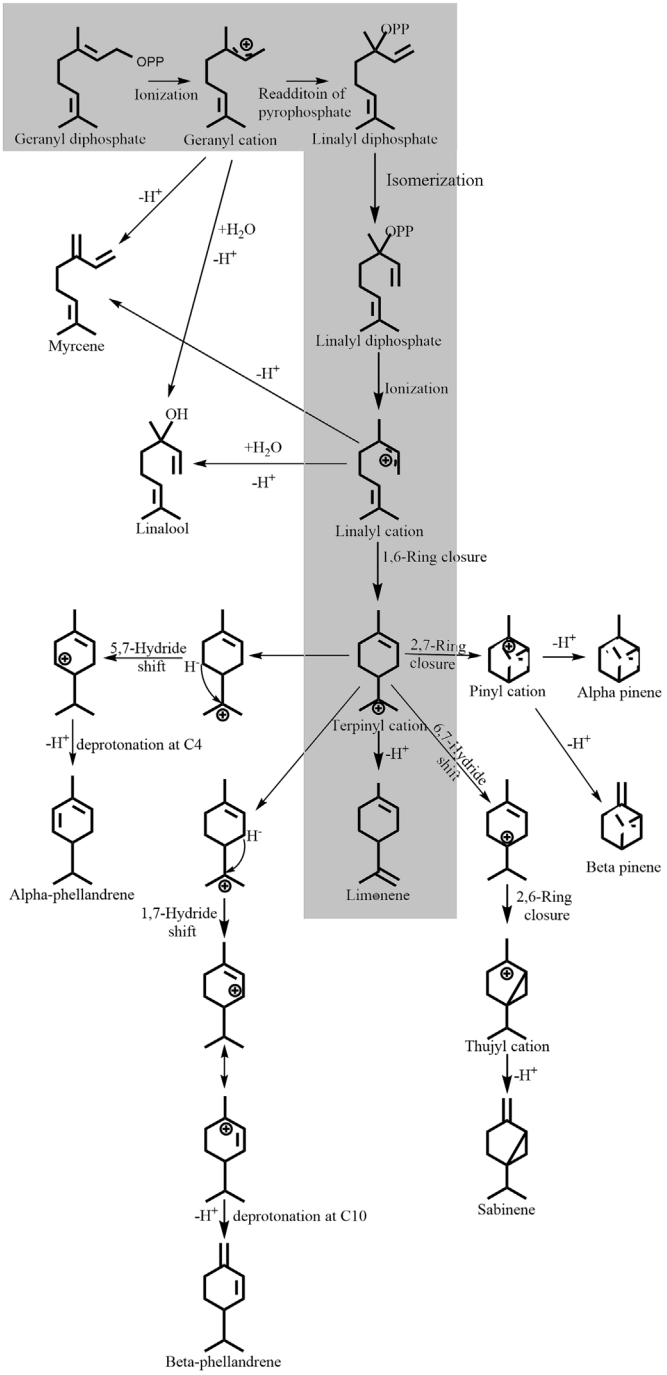

Fig. 1. Proposed reaction mechanism of limonene synthase.

conformation of the substrate or intermediate in the active site also significantly influence the product profile [7,8,11]. For example, in γ-humulene synthase there are two cyclization pathways diverged from two different conformation of carbocation, the trans-farnesyl cation and *cis*-farnesyl cation, with each producing different products [11].

LS is a typical monoterpene synthase. Hence we focus on this enzyme. Previous studies have revealed its cyclization mechanism (Fig. 1) [19–22]. But it is still unclear about the details of catalysis. Therefore, it is critical to identify residues that contribute to discrete steps within the reaction. In this study, we perform mutagenesis to investigate the function of Y573 in LS. Our data suggest that both the aromatic ring and hydroxyl group of this residue are critical for the production of limonene. In addition, dynamic simulation results show that the hydrogen bond between Y573 and D496 plays an essential role on the activity. Our research may provide important insights into the cyclization mechanism of limonene synthase.

2. Materials and methods

2.1. Chemicals

Tryptone and yeast extract were from Oxoid (UK). BL21 (DE3) and the Site-Directed Mutagenesis Kit were purchased from TransGen Biotech (China). Tris and Mops were from AMERSCO (USA). Dimethyl sulfoxide (DMSO) and glutathione were obtained from J&K Chemical (China). Glutathione Sepharose resin was from GE Healthcare Life Sciences (USA). Thrombin was from CALBIOCHEM (USA). Isopropyl β-D-thiogalactoside (IPTG), geranyl diphosphate (GPP), neryl diphosphate (NPP), phenylmethylsulfonyl fluoride (PMSF), EDTA and all other chemicals were obtained from Sigma Aldrich (USA).

2.2. Gene synthesis and mutagenesis

The gene sequences of *M. spicata* S-LS were synthesized by Sangon Biotech (China). The amino acid sequences from R58 to the C-terminus were subcloned into pGEX-4T-1 according to the study by Williams and colleagues [20]. Mutations were introduced using the site-directed mutagenesis kit and performed as previously described [17]. The PCR products were treated with DpnI and transformed into *E. coli* expression strain BL21(DE3). Correct mutants were identified by gene sequencing.

2.3. Protein expression and purification

For protein expression, cells were grown in LB media until it reached an OD_{600} of 0.6–0.8 and expression was induced by adding 0.5 mM IPTG at 20 °C for 16–20 h. For protein purification, cells were harvested by centrifugation and resuspended in buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.2 mM PMSF. After sonicated, Lysate was applied to glutathione Sepharose resin and eluted with 25 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA after on-column cleavage by thrombin. Protein purity was verified by SDS-PAGE.

2.4. The GC-MS assay

The reaction was carried out as previously described [17]. In brief, 400 μl reaction system contains 25 mM Mops (pH 7.0), 15 mM $MgCl_2$, 1.0 mM DTT, 10% (v/v) glycerol, 68.5 μM GPP and 1 μg protein. NPP solution was prepared by dissolving 10 mg NPP in 5 ml DMSO. Since there is still some undissolved compound, the up-limit of its concentration was estimated to be about 6.36 mM. While using NPP as the substrate, the amount of protein was 10 μg and NPP 636 μM per reaction. The reaction was run at 37 °C for 40 min and the same volume hexane was added to extract terpenes. The products were analyzed by GC-MS (Thermo Trace GC Ultra & ISQ) as previously described [17]. Monoterpenes were identified from mass spectra and GC retention times by comparing with available authentic standards (S-limonene, α-pinene and β-pinene) and spectra in libraries (β-phellandrene and sabinene). The proportion of each product was based on the ratio of the relative peak abundance. The relative activity is calculated by dividing the total peak area for terpene products of one particular enzyme by that of the wild type limonene synthase. The limitation of detectable terpenes by GC-MS analysis is estimated to 200 ng/mL. The origin 8.5 software is used for data analysis.

2.5. Dynamic simulation

Two structures were employed. They include the chain A of 2ONG and 2ONH, respectively. Only the protein and linalyl diphosphate (LPP) ligand were retained in the structure. Then the structure is solvated in a