Different reaction mechanisms for cis- and trans-prenyltransferases

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Abstract

Octaprenyl diphosphate synthase (OPPs) and undecaprenyl diphosphate synthases (UPPs) catalyze consecutive condensation reactions of farnesyl diphosphate (FPP) with 5 and 8 isopentenyl diphosphate (IPP) to generate C40 and C55 products with trans- and cis-double bonds, respectively. In this study, we used IPP analogue, 3-bromo-3-butenyl diphosphate (Br-IPP), in conjunction with radiolabeled FPP, to probe the reaction mechanisms of the two prenyltransferases. Using this alternative substrate with electron-withdrawing bromo group at the C2 position to slow down the condensation step, trapping of farnesol in the OPPs reaction from radiolabeled FPP under basic condition was observed, consistent with a sequential mechanism. In contrast, UPPs reaction yielded no farnesyl carbocation intermediate under the same condition with radiolabeled FPP and Br-IPP, indicating a concerted mechanism. Our data demonstrate the different reaction mechanisms for cis- and tran-prenyltransferases although they share the same substrates.

Keywords:
Isoprenoid
Prenyltransferase
Reaction mechanism
Sequential
Concerted

Isoprenoids are an extensive group of natural products with different carbon skeletons constructed from the five-carbon isopentenyl diphosphate (IPP) [1]. Over 55,000 isoprenoid compounds have been identified, which are responsible for a variety of biological functions in bacteria, archaea, and eukaryotes [2,3]. Isoprenoids are synthesized by a large group of enzymes named prenyltransferases. A class of prenyltransferases catalyze chain elongation of an allylic diphosphate substrate [e.g. farnesyl diphosphate (FPP)] with specific numbers of IPP via 1–4 condensation reactions to generate linear products with defined chain lengths [4,5]. C15 FPP itself is produced by coupling of two IPP with its isomer dimethylallyl diphosphate through the C10 geranyl diphosphate (GPP) catalyzed by farnesyl diphosphate synthase (FPPs) [6].

Based on the stereochemistry of the double bonds formed during IPP condensation reactions, these prenyltransferases are classified as trans- and cis-types. Octaprenyl diphosphate synthase (OPPs) that catalyzes the condensation reactions of FPP with 5 IPP is trans-type and its long-chain C40 product constitutes the side chain of ubiquinone [7,8]. On the other hand, undecaprenyl diphosphate synthase (UPPs) that catalyzes condensation reactions of FPP with 8 IPP is cis-type and its long-chain C55 product serves as a lipid carrier to transport the carbohydrates across the cell membrane for the biosynthesis of bacterial peptidoglycan [9,10]. Thus, UPPs can serve as a target for new antibiotics. Selective inhibitors of S. pyogenes UPPs and their antibacterial activities have been reported [11].

Cis- and trans-prenyltransferases may utilize different strategies for catalysis although they share the same substrates FPP and IPP. This is suggested by the lack of sequence similarity between the two groups of prenyltransferases [12,13]. The known crystal structures show that trans-prenyltransferases use two conserved DDXXD motifs to coordinate with two or three Mg2+ ions for binding with the diphosphate group of the allylic substrate [14–16], whereas an Asp in the conserved P-loop of cis-type prenyltransferases (D26 in E. coli UPPs) plays the Mg2+-chelating role [17–20]. Two possible mechanisms proposed for prenyltransferase reactions are (1) sequential ionization-condensation-elimination mechanism where allylic substrate releases its diphosphate to form a carbocation intermediate, which is attacked by IPP, and a proton (H+ for trans-type and H2 for cis-type) is removed from IPP C2 to form the adduct, and (2) concerted condensation-elimination mechanism where ionization of allylic substrate and condensation of IPP occur simultaneously (Supplementary material: Scheme 1) [21]. FPPs (a short-chain trans-type enzyme) reaction had been shown to proceed through a sequential mechanism [22]. However, the mechanism of cis-prenyltransferases was not clearly determined.

In this paper, we examined the mechanisms of long-chain trans-OPPs and cis-UPPs, by attempting to trap the farnesyl carbocation...
intermediate from FPP by using a synthetic IPP analogue with bromo to slow down the condensation step. The evidence of different mechanisms for the two types of prenyltransferases was obtained as reported herein.

**Materials and methods**

Chemicals. Radiolabeled [14C]IPP (55 mCi/mmol) and [3H]FPP (17 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Radiolabeled [14C]FPP (40–60 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. Thin layer chromatography (TLC) plates were purchased from Merck. Potato acid phosphatase (2 U/mg) was purchased from Roche Molecular Biochemicals. E. coli OPPs and UPPs were prepared as previously reported [23,24]. All reagents and solvents used in the organic synthesis were purchased from Sigma–Aldrich, Acros, and Fluka.

General methods. Proton and carbon NMR spectra are reported in parts per million downfield from internal Me$_3$Si, and phosphorus spectra in parts per million downfield from external phosphoric acid. NMR spectra were obtained in either CDCl$_3$ or D$_2$O. Silica gel column chromatography was performed on grade 60, 235–400 mesh silica gel. Nickel columns were prepared by adding 250 mg of Ni on grade 60, 235–400 mesh silica gel. A portion of reaction solution (33 L of 0.6 N NaOH to terminate the enzyme reaction. Octane was utilized to extract [3H]FOH resulted from the intermediate if there was, which was quantitated by scintillation counting.

**Analysis of reaction intermediate and products by TLC.** The reaction condition was 10 μM enzyme (OPPs or UPPs), 10 μM [14C]FPP, and 100 μM Br-IPP in buffer of 100 mM HEPES-KOH (pH 7.5), 0.5 mM MgCl$_2$, 50 mM KCl, and 0.1% Triton X-100. After incubating for 20 min, 100 μL reaction mixture was mixed with 200 μL NaOH (0.6 N) to terminate the enzyme reaction and the [3H]FOH if formed was extracted with equal volume of n-octane (radiolabeled polypropenyl diphosphates were in the aqueous phase). The octane solution after evaporation to reduce volume was spotted on a reversed-phase TLC plate, and then eluted with acetone/water (18:2) for 200 min. The 20% propanol solution containing 4.4 U/}

of 10% CH$_3$CN in 25 mM NH$_4$HCO$_3$ to 40% CH$_3$CN over 50 min. The final product (2) was obtained in 24% yield as a white solid. 1H NMR (400 MHz, D$_2$O) $\delta$ 2.74 (t, $J_{FH} = 6$ Hz, 2H), 4.05 (dt, $J_{FH} = 6.2$ Hz, $J_{HP} = 7.4$ Hz, 2H), 5.51 (s, 1H), 5.75 (s, 1H); 13C NMR (400 MHz, D$_2$O) $\delta$ 41.46 (d, $J_{CP} = 7$ Hz), 63.45 (d, $J_{CP} = 5$ Hz), 119.13, 129.61; 31P NMR (400 MHz, D$_2$O) $\delta$ 1.55, −7.83; HRMS: m/z calculated for C$_{4}$H$_{8}$BrO$_{7}$P$_{2}$ ($M^+$) 308.8934, found 308.8776.

**Attempt of trapping farnesyl cabocation intermediate using radiolabeled FPP and Br-IPP.** Intermediate trapping in the OPPs and UPPs reactions was attempted in the presence of Br-IPP. In a reaction mixture containing 10 μM OPPs or UPPs, 100 μM Br-IPP, 0.5 mM MgCl$_2$, 50 mM KCl and 0.1% Triton X-100 in 100 mM Hepes-KOH (pH 7.5) at 25 °C, 0.5 μM [3H]FPP was added to initiate the enzyme reaction. A portion of reaction solution (33 μL) was withdrawn after 0, 5, 10, 15, 20, 40, 60, and 80 min and mixed with 67 μL of NaOH (0.6 N) to terminate the enzyme reaction. Octane was utilized to extract the [3H]FOH resulting from the intermediate if there was, which was quantitated by scintillation counting.

**Synthesis of 3-bromo-3-butenyl p-methylbenzenesulfonate (1).** Compound 1 was synthesized by following the general procedure of Davission et al. as shown in Scheme 2 (Supplementary material) [25]. 2.53 g (13.25 mmol) of crystallized p-toluenesulfonyl chloride and 1.94 g (15.9 mmol) of 4-((NN-dimethylamino)pyridine were dissolved in dichloromethane (0.2 M in p-toluenesulfonyl chloride) with magnetic stirring under nitrogen. To this solution was added 2.0 g (13.25 mmol) of 3-bromo-3-buten-1-ol, and the reaction mixture was stirred overnight. The mixture was poured into a 100-fold excessive volume of hexane, and the resulting precipitate was removed by filtration. The filtrate was concentrated at reduced pressure, and the product was purified by column chromatography to afford 3.23 g (80%) of colorless oil. 1H NMR (400 MHz, CDCl$_3$) $\delta$ 2.47 (s, 3H), 2.76 (t, $J = 6$ Hz, 2H), 4.21 (t, $J = 6$ Hz, 2H), 5.50 (s, 1H), 6.47 (s, 1H), 7.26 (d, $J = 8$ Hz, 2H), 7.80 (d, $J = 8$ Hz, 2H).

**Synthesis of 3-bromo-3-butenyl diposphate (Br-IPP) (2).** To 3-bromo-3-butenyl p-methylbenzenesulfonate (10 mg, 0.03 mmol) was added 67.7 mg (0.075 mmol) of tris(tetrabutylammonium)hydrogen diphosphate in acetonitrile (0.5–1.0 M) and stirred overnight. The resulting material was converted to the ammonium form with 10 equivalents of resin, and after lyophilization the resulting powder was purified by reversed-phase HPLC on C8 column. Preparative-scale chromatography was performed on Agilent HP-1100 liquid chromatography. 25 mM NH$_4$HCO$_3$, pH 7.5, was used to dissolve samples and as the aqueous component in reversed-phase HPLC. All solvents were filtered and degassed before use, and samples were passed through a 0.45 μm filter before injection. Br-IPP (2) was purified by reversed-phase HPLC on a 250 x 10 mm Thermo C8 column and eluted with a linear gradient.
mL acidic phosphatase, 0.1% Triton X-100, and 50 mM sodium acetate (pH 4.7) was used to convert \([14C]FPP\) to \([14C]FOH\) that was used as a standard. The TLC plate with radiolabeled products was analyzed by autoradiography using a bioimaging analyzer (Fujifilm BAS-1500).

**Results**

No intermediate was trapped in OPPs and UPPs reactions with only radiolabeled FPP

We first attempted to trap the possible farnesyl carbocation intermediate by incubating \([3H]FPP\) with OPPs or UPPs. After incubation, the mixture was quenched with base and octane was used to extract the \([3H]FOH\) if formed. As shown in Fig. 1A and B for OPPs and UPPs reactions, respectively, no radioactivity could be obtained in the octane layer.

**Synthesis of Br-IPP**

The failure of intermediate trapping may be due to the absence of the other substrate IPP. To maximize the possibility of trapping farnesyl carbocation intermediate, we used Br-IPP with an electron-withdrawing bromo group attached to the C3 of IPP to slow down the condensation step by destabilizing the formed carbocation. For the synthesis of Br-IPP, a commercially available 3-bromo-3-buten-1-ol was converted to the tosylate species 1, which was then reacted with the diphosphate salt to yield Br-IPP (2) (Supplementary material: Scheme 2). This substrate analogue showed a significantly reduced activity as shown below.
Farnesyl carbocation intermediate was trapped in OPPs reaction, but not in UPPs reaction with radiolabeled FPP and Br-IPP

For trapping farnesyl carbocation intermediate, Br-IPP (100 μM) was added with the [3H]FPP substrate (0.5 μM) into the reaction mixtures of OPPs and UPPs, respectively, each containing 10 μM enzyme. In the presence of Br-IPP, radioactivity associated with [3H]FOH resulted from the farnesyl carbocation intermediate in the reaction of OPPs was detectable in the octane layer (Fig. 2A). The maximal quantity of the intermediate was observed after 15-min single-turnover reaction. However, the radiolabeled intermediate was not detectable for UPPs under the same condition (Fig. 2B).

Discussion

Both OPPs and UPPs catalyze multiple IPP condensation reactions, leading to long-chain products. With normal substrate IPP, no farnesyl carbocation can be trapped in both reactions. However, when the condensation is slowed down by Br-IPP, the farnesyl carbocation intermediate is formed in the OPPs reaction, giving direct proof for the sequential ionization-condensation-elimination mechanism for trans-prenyltransferases. In contrast, no such intermediate was trapped in UPPs reaction under the same reaction conditions with Br-IPP, indicating it may undergo a concerted reaction. As illustrated in Fig. 4, FPP binds to UPPs first and then IPP as a Mg2+-complex binds to the active site with D26, and then the Mg2+ is transferred to the diphosphate of FPP as previously demonstrated [20]. However, as shown in this study, the diphosphate of FPP is released and IPP attacks the farnesyl carbocation intermediate simultaneously without accumulation of the intermediate. Since Br-IPP can slow down the UPPs reaction, a cationic character on C3 of IPP must develop after the condensation (also shown in Fig. 4). Elimination of the H3 proton leads to a new cis-double bond to neutralize the carbocation.

Unlike UPPs, the active site of OPPs bears negative charges near the C1 of FPP, which may induce the formation of the farnesyl carbocation intermediate. As shown in the crystal structures of FPPs,
the side chain oxygen atoms of Thr203 and Gln241 and the main chain carbonyl of Lys202 are oriented with their negative dipoles directed toward the allylic carbocation-binding site [16]. The derived information of intermediate as presented in this study is useful for drug design. In fact, the nitrogen atom in the FPPs inhibitors (e.g. zoledronate) next to the bisphosphonate group was designed to mimic the cationic character of the intermediate [26]. However, in UPPs, this strategy may not be useful without definite formation of the carbocation intermediate. As shown by the QSAR analysis based on the crystal structures of the bisphosphonate inhibitors binding with UPPs, the binding affinity is solely determined by the bisphosphonate head and the hydrophobic tail [27]. Taken together, our data enhance our understanding on the mechanisms of two different prenyltransferases, particularly UPPs, and facilitate the drug discovery against this enzyme.

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Appendix A. Supplementary data


References