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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 C_{40} and C_{55} 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 C3 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.

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]. C_{15} FPP itself is produced by coupling of two IPP with its isomer dimethylallyl diphosphate through the C_{10} 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 C_{40} 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 C_{55} 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. peumoniae* 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 Mg^{2+} 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 Mg^{2+}-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_R for *trans*-type and H_S 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

Abbreviations: IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; FPPs, farnesyl diphosphate synthase; OPPs, octaprenyl diphosphate synthase; UPPs, undecaprenyl diphosphate synthase; UPP, undecaprenyl diphosphate; TLC, thin layer chromatography; FOH, farnesol; Br-IPP, 3-Bromo-3-butenyl diphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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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 [^{14}C]IPP (55 mCi/mmol) and [^{3}H]FPP (17 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Radiolabeled [^{14}C]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* UPPs and OPPs 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_4Si, and phosphorous spectra in parts per million downfield from external phosphoric acid. NMR spectra were obtained in either $CDCl_3$ or D_2O. Silica gel column chromatography was performed on grade 60,235-400 mesh silica gel (Merck), and TLC was performed on silica gel 60 F-254 glass plates (Merck). Silica TLC plates were visualized under UV light, by iodine, or by dipping in a 10% solution of phosphomolybdic acid in methanol followed by heating. Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated.

Attempt of trapping farnesyl carbocation intermediate in the OPPs and UPPs reactions using radiolabeled FPP. In a reaction mixture containing 5 μM OPPs or UPPs, 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 of [^{3}H]FPP was added to initiate the enzymatic 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 0.6 N NaOH to terminate the enzyme reaction. Octane was utilized to extract [^{3}H]FOH (farnesol) under basic condition if formed, while the substrate [^{3}H]FPP was still in the aqueous phase.

*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-(*N*,*N*-dimethy1amino)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. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.47 (s, 3H) 2.76 (t, J = 6 Hz, 2H), 4.21 (t, J = 6 Hz, 2H), 5.50 (s, 1H), 5.67 (s, 1H), 7.36 (d, J = 8 Hz, 2H), 7.80 (d, J = 8 Hz, 2H).

*Synthesis of 3-bromo-3-butenyl diphosphate (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_4HCO_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 × 10 mm Thermo C8 column and eluted with a linear gradient of 10% CH_3CN in 25 mM NH_4HCO_3 to 40% CH_3CN over 50 min. The final product (**2**) was obtained in 24% yield as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 2.74 (t, J = 6 Hz, 2H), 4.05 (dt, $J_{H,H}$ = 6.2 Hz, $J_{H,P}$ = 7.4 Hz, 2H), 5.51 (s, 1H), 5.75 (s, 1H); ^{13}C NMR (400 MHz, D_2O) δ 41.46 (d, $J_{c,p}$ = 7 Hz), 63.45 (d, $J_{c,p}$ = 5 Hz), 119.13, 129.61; ^{31}P NMR (400 MHz, D_2O) δ 1.55, −7.83; HRMS: m/z calculated for $C_4H_8BrO_7P_2$ (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 [^{3}H]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 [^{3}H]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 [^{14}C]FPP, 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 [^{14}C]FOH if formed was extracted with equal volume of n-octane (radiolabeled polyprenyl 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/

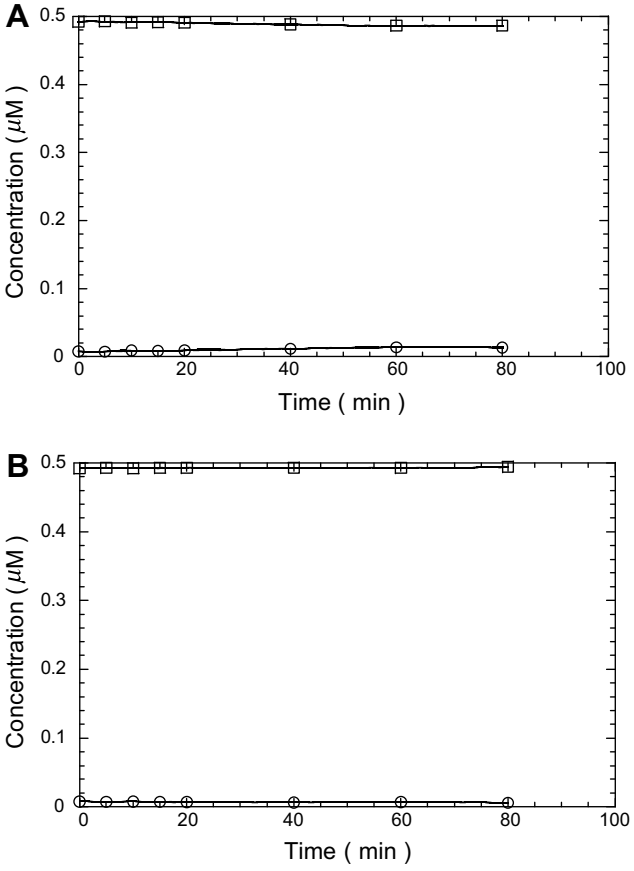

Fig. 1. Time courses of the incubation of 0.5 μM [^{3}H]FPP (□) with 5 μM OPPs (A) and UPPs (B) in the absence of Br-IPP. No radiolabeled [^{3}H]FOH (○) extractable by octane was found.