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Reviews

Structures, mechanisms and inhibitors of undecaprenyl diphosphate synthase: A *cis*-prenyltransferase for bacterial peptidoglycan biosynthesis

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ABSTRACT

Isoprenoids are an intensive group of compounds made from isopentenyl diphosphate (IPP), catalyzed by prenyltransferases such as farnesyl diphosphate (FPP) cyclases, squalene synthase, protein farnesyltransferases and geranylgeranyltransferases, aromatic prenyltransferases as well as a group of prenyltransferases (*cis*- and *trans*-types) catalyzing consecutive condensation reactions of FPP with specific numbers of IPP to generate linear products with designate chain lengths. These prenyltransferases play significant biological functions and some of them are drug targets. In this review, structures, mechanisms, and inhibitors of a *cis*-prenyltransferase, undecaprenyl diphosphate synthase (UPPS) that mediates bacterial peptidoglycan biosynthesis, are summarized for comparison with the most related *trans*-prenyltransferases and other prenyltransferases.

Contents

1. Introduction

Isoprenoids are natural products composed of 5-carbon isopentenyl diphosphate (IPP) as the building block [1,2]. These compounds are ubiquitous in Eukarya, Bacteria and Archaea to serve a variety of different biological functions [3]. As illustrated in Fig. 1, for the biosynthesis of isoprenoid natural products, IPP is first converted to its isomer dimethylallyl diphosphate (DMAPP) by the isomerase [4]. This allylic compound can condense with one and two molecules of IPP to form geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) by GPP synthase (GPPS) and FPP synthase (FPPS), respectively [5,6]. FPP serves as an outlet point leading to a variety of different isoprenoid compounds. Its cyclization by cyclases leads to cyclic sesquiterpenes such as pentalenene, trichodiene, and epi-aristolochene [7–9]. Two FPP condense to form squalene catalyzed by squalene synthase (SQS) and then to cholesterol and some hormones in animals [10]. FPP can react with one IPP to generate geranylgeranyl diphosphate (GGPP) by GGPP

Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; SQS, squalene synthase; GGPP, geranylgeranyl diphosphate; DMATS, dimethylallyl transferase; GGPPS, geranylgeranyl diphosphate synthase; DPPS, decaprenyl diphosphate synthase; UPPS, undecaprenyl diphosphate synthase; HexPPS, hexaprenyl diphosphate synthase; HepPPS, heptaprenyl diphosphate synthase; OPPS, octaprenyl diphosphate synthase; Br-IPP, 3-bromo-3-butenyl diphosphate.

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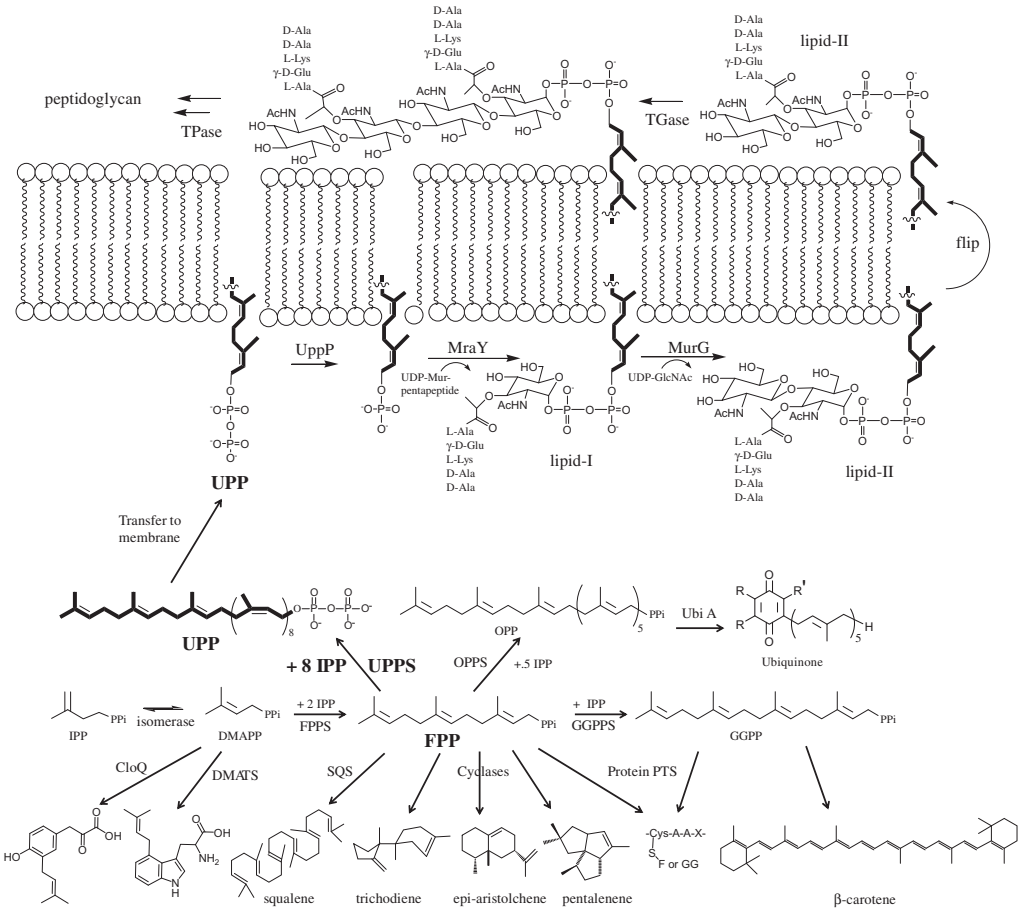

Fig. 1. Prenyltransferases discussed in this review and the biosyntheses of natural isoprenoids they are involved. The UPP highlighted serves as lipid carrier for bacterial peptidoglycan biosynthesis.

synthase (GGPPS) [11,12]. GGPP is the precursor of natural products such as ether-linked lipids in archeon, chlorophylls, α-tocopherol or longer prenyl diphosphates used in quinine biosynthesis, ent-kaurene, taxadiene, phytoene and carotenoids. Among them, carotenoids and retinoids containing highly conjugated structures for absorption of light are involved in the light-sensitive elements. Both FPP and GGPP can be added to signaling proteins on a conserved cysteine located in a C-terminal CaaX motif with "a" often an aliphatic residue and "X" Ser, Met, Ala, or Gln by protein prenyltransferases [13,14]. Prenylation (i.e. farnesylation or geranylgeranylation) of Ras, Rab, nuclear lamins, trimeric G-proteins γ subunits, protein kinases, and small Ras-related GTP-binding proteins for anchoring them into the cellular membranes is essential for signal transduction pathways [15]. Aromatic prenyltransferases such as DMAPP transferase (DMATS) and CloQ transfer prenyl moieties (e.g. DMAPP) onto aromatic acceptor molecules, forming covalent bonds between C1 or C3 of the isoprenoid moiety and an atom of the aromatic ring [16]. These products are primary and secondary aromatic metabolites in plant, fungi, and bacteria [17,18].

FPP is also a starting material for a group of prenyltransferases, each synthesizing a linear polymer with designate chain length via condensation reactions with a specific number of IPP [19,20]. Based on the stereochemistry of double bonds formed during IPP condensation, these prenyltransferases are classified as *trans*- and *cis*-types. *trans*-Prenyltransferases generate products with chain lengths ranging from C_{10} to C_{50}, whereas *cis*-prenyltransferases produce larger polymers including C_{50} by decaprenyl diphosphate synthase (DPPS), C_{55} by undecaprenyl diphosphate synthase (UPPS), C_{120} by a *cis*-prenyltransferase from *Arabidopsis thaliana* [21], to almost unlimited chain lengths by rubber prenyltransferases [22] with an exception of a short-length *cis*-type FPPS found in *Mycobacterium tuberculosis* [23]. The C_{30}, C_{35}, C_{40}, C_{45}, and C_{50} products generated by hexaprenyl diphosphate synthase (HexPPS) [24], heptaprenyl diphosphate synthase (HepPPS) [25], octaprenyl diphosphate synthase (OPPS) [26], solanesyl diphosphate synthase (SPPS), and DPPS, respectively, constitute the side chains of ubiquinone and menaquinone in different species.

For the reasons of using prenyltransferases as drug targets, oncogenic Ras farnesylated as mutant forms has been detected in approximately 30% of all human cancers [27] and oncogenic H-Ras is known for its Akt-1 activation and transforming activity [28]. Geranylgeranylated Rho GTPases play key roles in the recognition of the actin cytoskeleton and the control of gene transcription [29–31]. Bis-geranylgeranylated Rab GTPases regulate vesicular trafficking and exocytosis [32,33]. Therefore, protein farnesyltransferases have been regarded as anti-cancer targets [34–36]. Furthermore, inhibitors of prenylation of Ras and other G-proteins have been developed for therapeutics in restenosis, angiogenesis and osteoporosis [37]. FPPS for synthesizing FPP is the drug target of bisphosphonate drugs for preventing the bone loss associated with osteoporosis, Paget's disease, hypercalcemia, and metastatic bone disease [38] and GGPPS may serve as a target for some cases [39].

On the other hand, the C_{55} product of bacterial UPPS serves as a lipid carrier in cell wall peptidoglycan biosynthesis as shown in

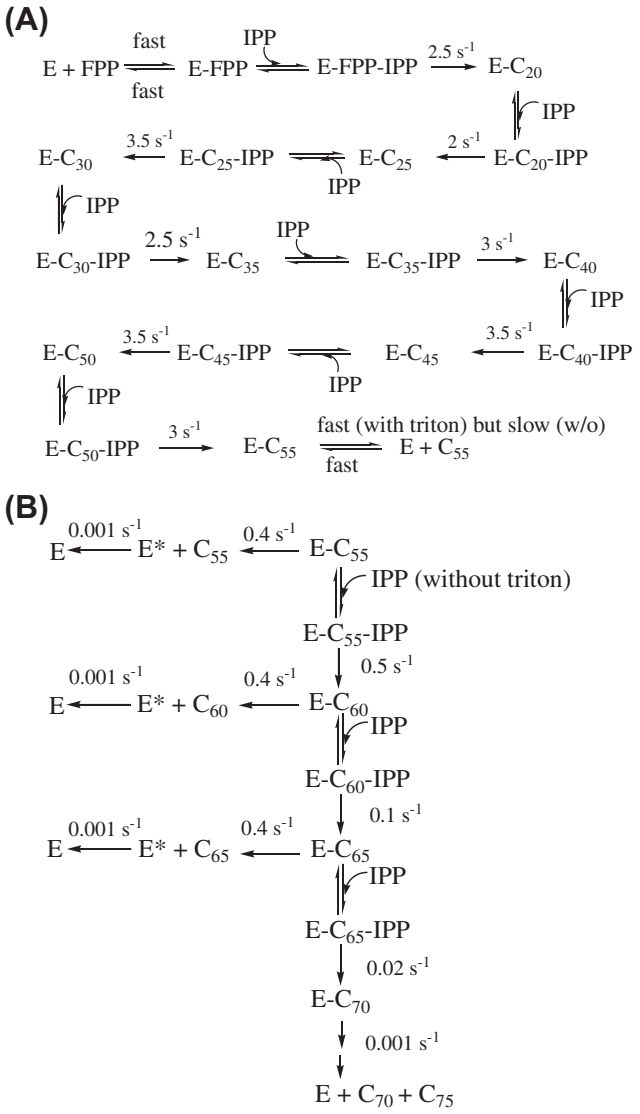

Fig. 2. Kinetic schemes of UPPS reaction up to product formation (A) and after C_{55} product formation in the absence of Triton (B).

Fig. 1 [40]. In this process, UPP first undergoes dephosphorylation and reacts with muramyl-pentapeptide to form lipid I which reacts with N-acetylglucosamine to form lipid II. Lipid II is transported across cell membrane and catalyzed by transglycosylase and transpeptidase to form peptidoglycan. Due to this pivotal role in bacterial peptidoglycan biosynthesis, UPPS serves as an antibacterial target. However, it should be cautious that its homolog dehydrodolichyl diphosphate synthase in human is responsible to make ~C_{100} dolichols for glycoprotein biosynthesis in ER [41].

Compared to the well studied *trans*-prenyltransferase, structural and mechanistic studies have just been performed in *cis*-prenyltransferases, particularly UPPS, as summarized in a recent review [42] since its gene cloning about a decade ago [43]. The complex kinetic scheme for the multiple IPP condensation steps in UPPS reaction (Fig. 2) has been solved. Structures of different forms of UPPS with or without ligands reveal conformational changes upon substrate binding, catalysis and product release (Fig. 3). A disordered loop in *Escherichia coli* UPPS, invisible in crystal structures without ligand is essential for catalysis. In contrast to *trans*-prenyltransferases, UPPS has been shown to utilize concerted mechanism for IPP condensation (Fig. 4). Inhibitors of UPPS from high throughput screening and rational design have been reported (Fig. 5). These are described in more details herein.

1.1. Kinetic scheme of UPPS reaction

UPPS catalyzes eight consecutive condensation steps of IPP with FPP to form C_{55} final product. There should be C_{20}–C_{50} intermediates whether detectable or not in the whole reaction. It had been a challenge for kinetic measurements of long-chain lipid synthases catalyzed multiple-step reactions and previous kinetic characterization on UPPS was based on the radiolabeled IPP consumption or butanol-extractable radiolabeled product formed with time. Each step of the UPPS reaction was never characterized until the work reported by Pan et al. [44]. To study the pre-steady-state UPPS kinetics, single-turnover with excessive enzyme over the substrate FPP and sufficient quantity of [^{14}C] IPP was performed and the reaction was stopped using rapid-quench instrument in sub-second time scale. The C_{20}–C_{55} ^{14}C-labeled intermediates and final product were hydrolyzed (removing diphosphate), separated and analyzed using reverse-phase TLC. From the time courses, the kinetic constants for all the IPP condensation steps were

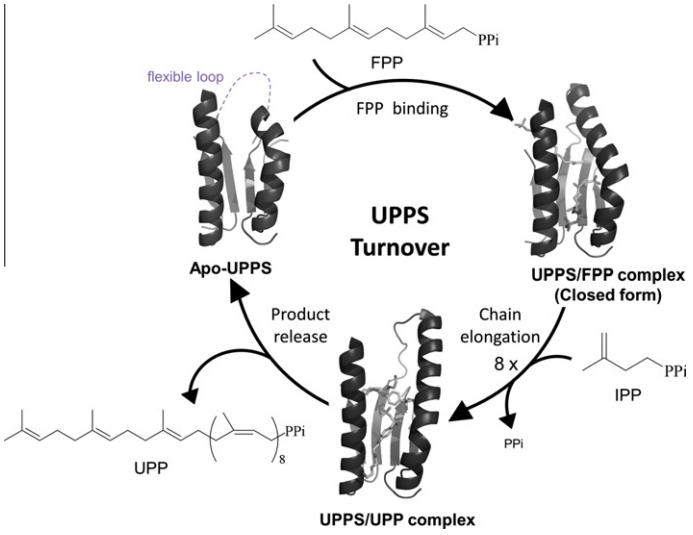

Fig. 3. Conformational changes of UPPS during its reaction. Apo-UPPS has a flexible loop without visible electron density in crystal structure, and the binding of FPP and product-like Triton X-100 shift UPPS to closed form and open form, respectively.

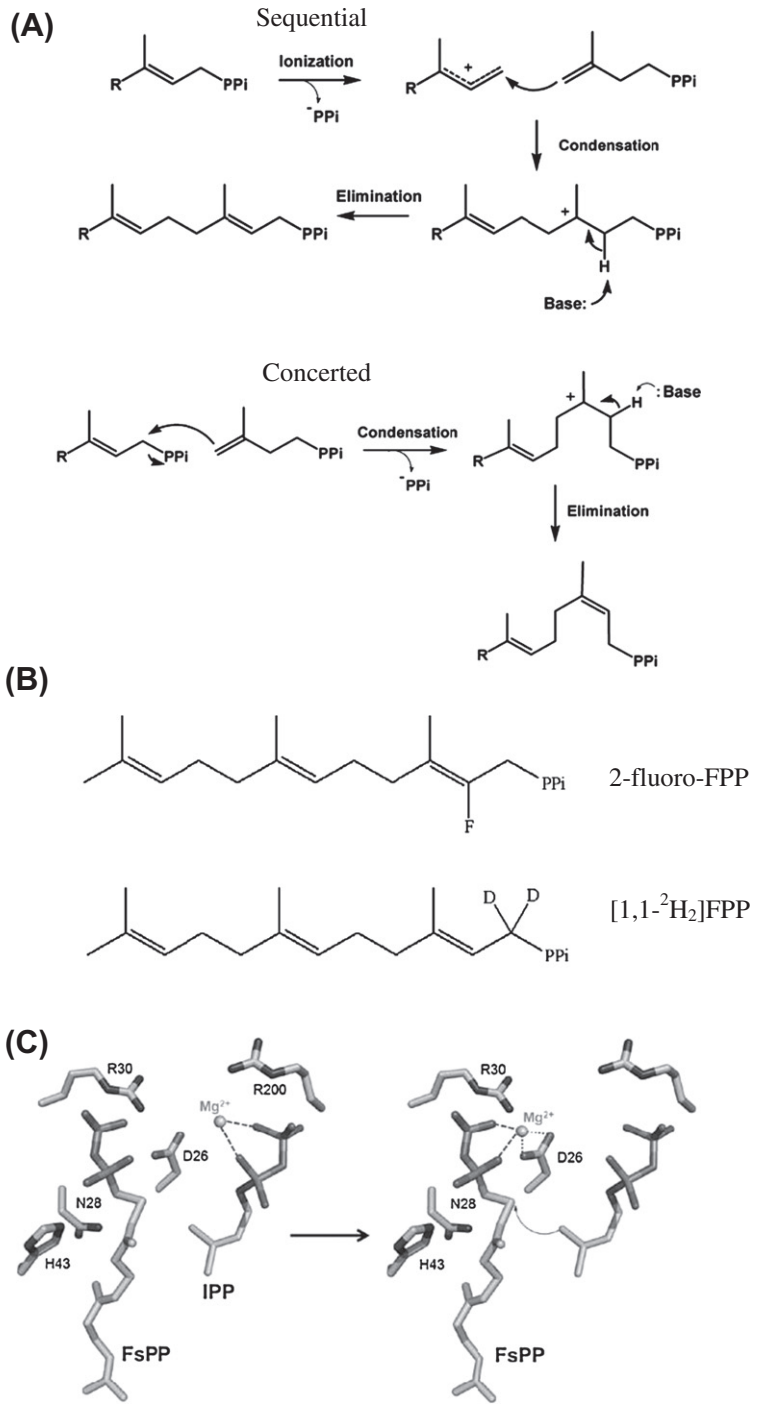

Fig. 4. Mechanism of IPP condensation catalyzed by UPPS. (A) Two possible mechanisms for IPP condensation reactions. (B) The FPP analogs used to test the mechanisms. (C) The structures of UPPS (D26A in the left and wt in the right) in complexation with FsPP and Mg^{2+}–IPP support the concerted mechanism.

obtained using kinetic simulation program (KINSIM), which are approximately equal to 2.5 s^{-1} (Fig. 2A). The product release is rate limiting since the C_{20}–C_{55} time courses under single-turnover condition are similar in the presence or absence of 0.1% Triton X-100 detergent but longer products are also detected in the absence of Triton indicating C_{55} is retained in the active site for further elongation, whereas the k_{cat} is increased by 190-fold under steady-state condition by 0.1% Triton X-100. The multiple-turnover experiments further support slow product release in the absence of Triton by following a burst kinetics and the KINSIM fitting suggests slow product release composed of a 0.4 s^{-1} product dissociation followed by a much slower 0.001 s^{-1} conformational change (Fig. 2B) to rationalize the accumulation of C_{60} and longer species as final products and to match the k_{cat} value (if C_{60} is not dissociated with at least 0.4 s^{-1}, it would be converted to C_{65} at the estimated rate constant of 0.5 s^{-1}).

In comparison, the long-chain *trans*-type OPPS reaction proceeds through C_{20}–C_{35} intermediates as shown by reverse-phase TLC [45]. The kinetic constants for IPP condensation steps are approximately equal to 2 s^{-1}, similar to that (2.5 s^{-1}) of the UPPS catalyzed reaction, indicating similar activation energy. However, as discussed below, the IPP condensation reaction mechanism of

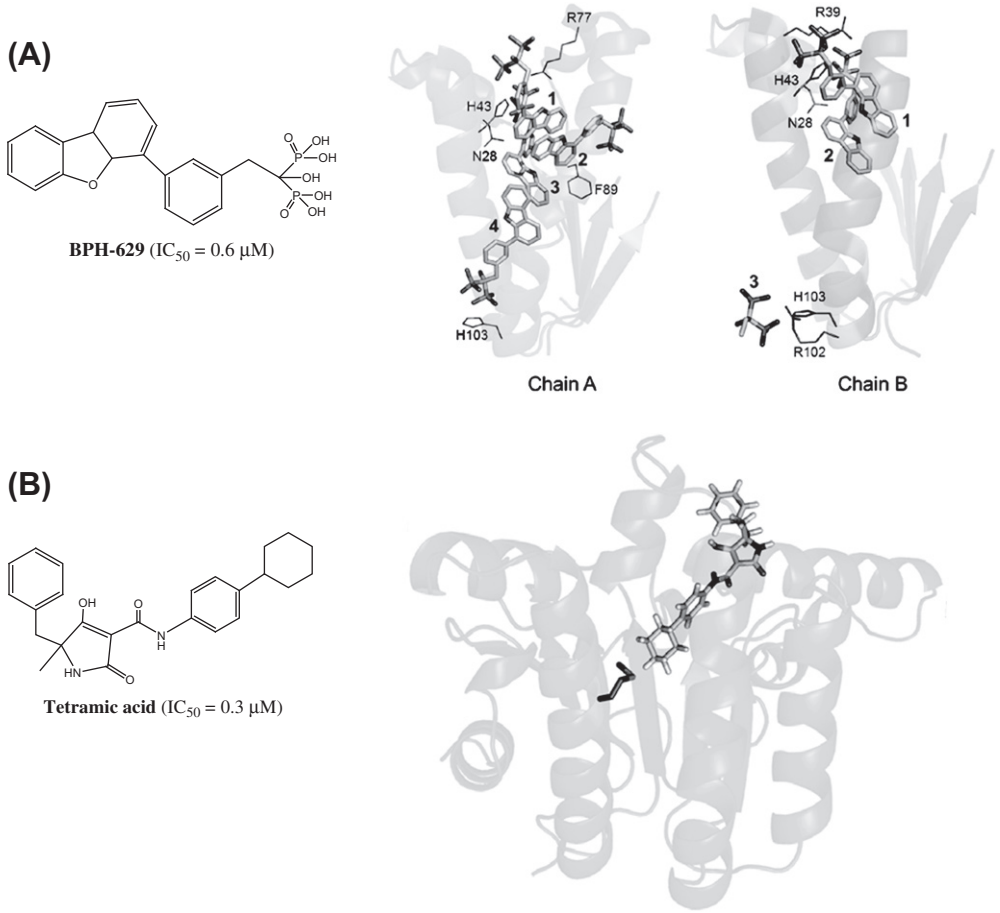

Fig. 5. Inhibitors of UPPS. (A) bisphosphonate with bulky aromatic side chain and its complexed structures with UPPS. (B) Tetramic acid (from Ref. [68]) and its complexed structure with *S. pneumoniae* UPPS (entry Q97SR4) generated by computer modeling using Discovery Studio Modeling 1.2 SBD based on *M. luteus* B-P 26 UPPS structure (PDB entry 1F75) as template from SWISS-MODEL Repository.

trans-prenyltransferases is sequential, but that of the *cis*-prenyltransferase (UPPS) is concerted. Also Triton and other detergents tested were not able to activate OPPS reaction, suggesting product release as rate limiting step even in the presence of detergent.

1.2. Product dissociation rate constants

To confirm the product dissociation rates in the absence and presence of 0.1% Triton X-100, which should be slower and faster, respectively, stopped-flow instrument was used with a fluorescent FPP analog, 7-(2,6-dimethyl-8-pyrophospho-2,6-octadienyloxy)-8-methyl-4-trifluoromethyl-chromen-2-one named TFMC-GPP in short, to probe the product dissociation rate constants. This fluorescence probe with coumarin attached to GPP, resembling FPP structure can bind to the UPPS active site, leading to decreased fluorescence [46]. The titration curve showed 1:1 binding stoichiometry with UPPS. Using this probe as a competing agent to displace UPP from the active site, the product dissociation rate constant was measured to be 0.5 s^{-1}, consistent with the prediction by KINSIM, in the absence of Triton. Using reduced-form Triton X-100 that contains cyclohexyl rather than benzene in the structure to prevent fluorescence interference, the product dissociation rate constant was measured to be 15.7 s^{-1}, showing faster product release in the presence of 0.1% detergent [47]. Therefore, IPP condensation becomes rate limiting in the presence of 0.1% Triton X-100, whereas product release is rate limiting in the absence of detergent.

1.3. Conformational changes

To look for the evidence for conformational changes in the UPPS reaction, several X-ray crystal structures of UPPS with and without ligands have been solved and compared (Fig. 3). UPPS without ligand is flexible and a loop containing aa 72–83 is invisible in the crystal structure, indicating it is highly disordered [48]. However, when FPP or a small ligand is bound, UPPS adopts the closed form with α3 more kinked towards the active site [49]. Based on the complexed structure, the diphosphate head group of FPP is bound to the backbone NHs of Gly29 and Arg30 as well as the side chains of Asn28, Arg30 and Arg39 through hydrogen bonds. The hydrocarbon moiety of FPP is bound with hydrophobic amino acids including Leu85, Leu88, and Phe89, located on α3 helix. In this case, no metal is required for FPP binding, although Mg^{2+} is absolutely needed for catalysis. When UPPS was crystallized with high concentration of Triton X-100, two molecules of Triton X-100 were clearly seen in the electron density maps of UPPS structure. The first Triton molecule (T1) occupies the lower or inner part of the tunnel, while the second Triton molecule (T2) binds to the upper or outer part [50]. The protein UPPS with both Tritons bound (resembling UPP product) adopts an open conformation with α3 helix more straight and elongated (open form). The inter-conversion between closed and open forms from substrate binding to product release was observed using steady-state and stopped-flow experiments [51]. It was shown that FPP binding quenches the fluorescence of Trp91 in the α3 helix, which moves toward the active site during substrate binding and results in a closed conformation to provide

better interaction of UPPS with the substrate. After the reaction, the crowding prenyl chain of the product pushes the UPPs structure to an open conformer for product release.

By comparing the structures of UPPS in apo form, closed form and open form, the most obvious differences occur in the positions of α3 and the loop connecting α3 and β3, which seem to regulate the conformational changes (see Fig. 3). To show the importance of the loop in conformational changes and identify which form (open or closed) is catalytically active, the UPPS mutants with amino acids inserted into or deleted from the loop to make the loop longer and shorter, respectively, were constructed, their kinetic properties were measured and 3-D structures were solved [52]. The Ala-insertion mutant fails to display the intrinsic fluorescence quenching upon FPP binding and its crystal structure reveals only the open form. The loop-shortening mutants with deletion of V82 and S83 or S72 also adopt an open form with the loop stretched and β3 redirected closer to α3 due to the shortening of loop. Both mutant UPPS showed 10^4-fold reduction in k_{cat}, indicating open form has almost abolished activity and the closed from is catalytically active.

1.4. Intermediate in each IPP condensation of UPPS reaction and the mechanism

Two possible mechanisms have been proposed for the prenyltransferase catalyzed condensation reactions: (1) a sequential ionization–condensation–elimination mechanism where allylic substrate (GPP or FPP) releases its diphosphate to form a carbocation intermediate, which is attacked by IPP to form the second carbocation intermediate, and a proton is then removed from the C2 position of IPP moiety to form the adduct and (2) a concerted condensation–elimination mechanism where ionization of the allylic substrate and condensation of IPP occur simultaneously (see Fig. 4A for the two possible mechanisms) [53]. While sharing the same substrates FPP and IPP, *cis*- and *trans*-prenyltransferases may utilize different mechanisms. The first evidence came from the study using 3-bromo-3-butenyl diphosphate (Br-IPP) as an alternative substrate, which has an electron-withdrawing bromo (Br) replacing methyl group to destabilize the second carbocation intermediate, thereby slowing down the condensation step to allow the trapping of farnesyl carbocation intermediate as farnesol (FOH) under basic condition if it is formed [54]. Br-IPP leads to detectable FOH in the *trans*-type GGPPS and OPPS reactions, but not in the UPPS reaction, suggesting UPPS may go through a concerted mechanism. Moreover, two substrate analogs, 2-fluoro-FPP and [1,1-$^2H_2$]FPP as shown in Fig. 4B, were used to probe the intermediate and mechanism [47]. Fluorinated GPP substrate analogs have been used to probe the reaction mechanism of FPPS and the remarkably smaller kinetic constants using fluorinated GPP support the sequential mechanism and the formation of geranyl carbocation intermediate due to destabilization of intermediate by the strong electron-withdrawing fluorine substituent(s) [55]. Isotope effect using deuterium FPP substrate was used to probe the change of hybridization of the FPP C1-carbon in the protein farnesyltransferase reaction [56]. However, in the single-turnover experiments, the TLC analysis showed the rate of 2-fluoro-FPP condensing with IPP is similar to that of FPP, supporting the concerted condensation–elimination mechanism for UPPS [47]. The 60-fold smaller k_{cat} under steady-state condition may be due to slower release rate of the 2-fluoro-UPP product, as confirmed by the stopped-flow experiments. Moreover, the deuterium secondary KIE of 0.985 ± 0.022 measured for UPPS reaction using deuterium-FPP as substrate under steady-state condition is consistent with an associative transition state. This concerted mechanism is consistent with the structures of UPPS and its D26A mutant in complexation with Mg^{2+}, FsPP (inactive FPP thiol analog) and IPP (Fig. 4C), where a single Mg^{2+} was shown to chelate IPP in the absence of carboxylate in the D26A mutant (left) and chelate FPP in the presence of D26 side chain (right) [57]. Only one Mg^{2+} exists in the active site but two positions of Mg^{2+}, one associated with FPP and the other with IPP, were found in the structures. Therefore, a Mg^{2+} transfer mechanism is proposed for shift of Mg^{2+} from IPP to FPP via Asp26, thereby facilitating attacking of double bond of IPP on diphosphate-bearing carbon of FPP for condensation reaction. From these data, it is concluded that UPPS binds FPP first followed by Mg^{2+}–IPP as previously proposed [51] and Mg^{2+} is then transferred to the diphosphate of FPP to facilitate its dissociation and the simultaneous IPP attacking on the C1 position of FPP. Subsequent elimination of the H_S proton leads to a new *cis*-double bond to neutralize the carbocation and form the C_5-extended adduct for each IPP condensation reaction.

In contrast, other prenyltransferases, especially those use Asp-rich motif to coordinate a Mg^{2+} cluster for removing the diphosphate group of allylic substrates, proceed through a sequential mechanism with allylic carbocation intermediate. FPPS has been first shown to go through the sequential mechanism by trapping geranyl carbocation or using fluoro-analogs of the GPP substrate. Crystal structure of FPPS indicates three Mg^{2+} ions are chelated by the first DDXXD motif of the two conserved DDXXD motifs and the diphosphate oxygens of substrate thiol analog DMSPP [58]. FPP cyclases also possess Asp-rich motifs for catalyzing the farnesyl carbocation as the initial step of cyclization to form different products, depending on the shapes of the active sites [10–12]. Squalene synthase may also follow the mechanism with formation of farnesyl cabcation [13]. However, for the protein farnesyltransferases, there is no Asp-rich motif and Mg^{2+} was shown not essential but greatly (700-fold) accelerating the enzyme activity by coordinating the diphosphate leaving group to stabilize the transition state [59]. On the other hand, Zn^{2+} ion essential for the enzymes is used for activating the nucleophilic Cys in CaaX motif. Based on the secondary isotope effect, it was concluded that Ras FTase goes through a concerted mechanism with dissociative character using FPP and a peptide TKCVIF as substrates [56], in contrast to the fully dissociated carbocation in *trans*-prenyltransferases. For aromatic prenyltransferases, they are either Mg^{2+} required or not. The aromatic prenyltransferases involved in the biosynthesis of ubiquinones, menaquinones, and plastoquinones are membrane-bound, which have conserved (N/D)DXXD motifs for binding of isoprenoid substrates in the form of Mg^{2+} complexes. In contrast, the aromatic prenyltransferases for the biosynthesis of many microbial secondary metabolites are soluble enzymes without Asp-rich motifs. From sequence comparison, these Mg^{2+}-independent prenyltransferases can be further classified into two groups: dimethylallyl tryptophan synthase catalyzes the prenylation of indole moieties [60–62] and CloQ/NphB catalyze prenylation of phenols and phenazine derivatives [63,64]. Although Mg^{2+} is absent, these aromatic prenyltransferases are thought to go through the formation of a carbocation which is protected from solvent in a confined pocket.

1.5. Inhibitors of UPPS from rational design and high throughput screening

Bisphosphonates mimicking allylic diphosphate substrate have been used as inhibitors of FPPS and GGPPS. This kind of inhibitors with bulky aromatic side chains were found to inhibit UPPS [65] (as shown in Fig. 5A, left panel, for an example). As reveled by the UPPS–bisphosphonate complex structures (Fig. 5A, right panel), four bisphosphonate molecules are bound to the central cavity of the elongated active-site tunnel with three at the top and one at the bottom. One of them occupies the FPP site and none competes with IPP binding.

Based on the UPPS crystal structures, 58,635 compounds from Maybridge Chemical database were screened using the program GOLD V2.1 and an inhibitor HTS04781 which can inhibit *Helicobacter pylori* UPPS but not *E. coli* UPPS was found, giving the possibility of developing antibiotics specially targeting pathogenic *H. pylori* without killing the intestinal *E. coli* [66]. However, the inhibitor requires optimization for higher potency.

From high throughput screening, tetramic, tetronic acids and dihydropyridin-2-ones have been identified as inhibitors of *Streptococcus pneumoniae* UPPS (one example is shown in left panel of Fig. 5B, with IC_{50} = 0.3 μM and MIC = 0.5 μg/mL against *S. pneumoniae*) without inhibiting human FPPS [67]. By replacing cyclohexyl benzene ring with dibenzophenone in the inhibitor, the photoactivatable probe was used to covalently link with the UPPS upon UV irradiation and MASS finger printing was utilized to identify the bound region of this inhibitor, which is located at the Met49 in α3 helix [68]. The inhibitor is bound close to FPP site (allosteric) and probably alters the active closed conformation to almost inactive open form of UPPS (Fig. 5B, right panel).

2. Conclusion

In this review, the structures, mechanisms and inhibitors of UPPS, a *cis*-prenyltransferase for bacterial peptidoglycan biosynthesis, have been focused. Nevertheless, the structures, mechanisms and inhibitors of the most related *trans*-prenyltransferases and some other prenyltransferases, which are totally different from those of the *cis*-prenyltransferase, have been mentioned for comparison. The information summarized here serves as a useful background for further studying prenyltransferases. Judged from the large number (55,000) of isoprenoid natural products identified so far, there must be many prenyltransferases not been found and studied. Since natural isoprenoids play significant biological functions and may possess desired properties to be used as drugs or food supplements, identification of their synthetic gene clusters and studies of the prenyltransferases involved in their biosynthetic pathways are particularly worth pursuing for basic understanding and application.

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