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Kinetic Studies of *Micrococcus luteus* B-P 26 Undecaprenyl Diphosphate Synthase Reaction Using 3-Desmethyl Allylic Substrate Analogs

Keitaro FUJIKURA,[1] Yuji MAKI,[2] Norimasa OHYA,[2] Mikiya SATOH,[2] and Tanetoshi KOYAMA[1,†]

[1]*Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan*

[2]*Department of Biological and Material Chemistry, Faculty of Science, Yamagata University, Kojirakawa, Yamagata 990-8560, Japan*

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In order to investigate the substrate binding feature of undecaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26 with respect to farnesyl diphosphate and a reaction intermediate, (*Z*,*E*,*E*)-geranylgeranyl diphosphate, we examined the reactivity of artificial substrate analogs, 3-desmethyl farnesyl diphosphate and 3-desmethyl *Z*-geranylgeranyl diphosphate, which lack the methyl group at the 3-position of farnesyl diphosphate and *Z*-geranylgeranyl diphosphate, respectively. Undecaprenyl diphosphate synthase did not accept either of the 3-desmethyl analogs as the allylic substrate, indicating that the methyl group at the 3-position of the allylic substrate is important in the undecaprenyl diphosphate synthase reaction. These analogs showed different inhibition patterns in the *cis*-prenyl chain elongation reaction with respect to the reactions of farnesyl diphosphate and *Z*-geranylgeranyl diphosphate as allylic substrate. These results suggest that the binding site for the natural substrate farnesyl diphosphate and those for the intermediate allylic diphosphate, which contains the *cis*-prenyl unit, are different during the *cis*-prenyl chain elongation reaction.

Key words: prenyltransferase; isoprenoid biosynthesis; undecaprenyl diphosphate synthase; *cis*-prenyl chain elongating enzyme; substrate specificity

The carbon skeletons of isoprenoids (also called terpenes or terpenoids), approximately 30,000 structurally diverse compounds occurring in nature, are constructed by linear prenyl chain elongation of various chain lengths and stereochemistries catalyzed by prenyltransferases. Linear prenyl diphosphates are converted to such diverse isoprenoid compounds as steroids, carotenoids, glycosyl carrier lipids, prenyl quinones, and prenylated proteins in the biosynthetic pathway of isoprenoids. Prenyltransferases can be classified into two major subgroups, *cis*- and *trans*-types, according to the geometry of their products as determined by the distinct specificity of each enzyme.[1,2)]

Undecaprenyl diphosphate (UPP) synthase catalyses the consecutive *cis*-condensation of eight molecules of isopentenyl diphosphate (IPP) with (*E*,*E*)-farnesyl diphosphate (FPP) to generate UPP with *E*,*Z*-mixed stereochemistry. In bacteria, UPP is an essential intermediate in the biosynthesis of cell-wall peptidoglycan. After the first molecular cloning of the gene for UPP synthase of *Micrococcus luteus* B-P 26, the primary and crystal structures of the enzyme from *M. luteus* B-P 26 and *Eschericia coli* were determined.[3,4)] The primary and tertiary structures of the *cis*-type prenyltransferases have been found to be totally different from those of *trans*-type prenyltransferases.[4,5)] The molecular mechanism of *trans*-type prenyltransferases has been studied intensively.[6–15)] Nagaki *et al.* found that the presence of a methyl group at the 3-position of the allylic substrate is strictly essential for *trans*-type prenyltransferases using 3-desmethyl analogs, which lack the methyl group at the 3-position of allylic substrates.[16,17)] In contrast, the detailed mechanism of *cis*-type prenyltransferases has not yet been uncovered.

Our earlier kinetic studies on UPP synthase with respect to allylic substrates indicated that the binding site for the reaction intermediate (*Z*,*E*,*E*)-geranylgeranyl diphosphate (*Z*-GGPP), which has one *cis*-prenyl unit, is different to that for the intrinsic allylic substrate FPP.[18,19)]

In this study, in order to investigate the difference in the manner of allylic substrate recognition as between the FPP and the intermediate *Z*-GGPP of *cis*-type prenyltransferase UPP synthase, we synthesized 3-

† To whom correspondence should be addressed. Fax: +81-22-217-5621; E-mail: koyama@tagen.tohoku.ac.jp

Abbreviations: FPP, (*E*,*E*)-farnesyl diphosphate; *Z*-GGPP, (*Z*,*E*,*E*)-geranylgeranyl diphosphate; *E*-GGPP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; norFPP, 3-desmethyl (*E*,*E*)-farnesyl diphosphate; *Z*-norGGPP, 3-desmethyl (*Z*,*E*,*E*)-geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; DIBAL, diisobutylalminium hydride; DMS, dimethyl sulfide; NCS, *N*-chloro succinimide; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; UPP, undecaprenyl diphosphate

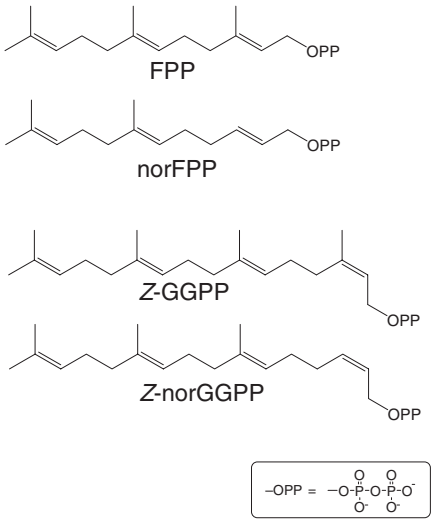

Fig. 1. Structures of the Intrinsic Substrate FPP and the Intermediate after One Prenyl Chain Elongation, *Z*-GGPP, and the Corresponding 3-Desmethyl Substrate Analogs, norFPP and *Z*-norGGPP.

desmethyl allylic substrate analogs 3-desmethyl FPP (norFPP) and 3-desmethyl *Z*-GGPP (*Z*-norGGPP), which lack the methyl group at the 3-position of FPP and *Z*-GGPP respectively, and examined the UPP synthase reactivity of these substrate analogs.

Materials and Methods

Materials. [1-^{14}C]IPP (2.12 TBq/mol) was purchased from GE Healthcare (Tokyo, Japan). Nonlabeled IPP, FPP, and *Z*-GGPP were synthesized according to the procedure of Davisson *et al.*[20] Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan), and Toyobo (Osaka, Japan). Potato acid phosphatase was from Sigma (Tokyo, Japan). *E. coli* B strain BL21 (DE3) was used as the host for expression of the target gene. Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook *et al.*[21] Precoated reversed phase thin layer chromatography (TLC) plates were purchased from Merck (Tokyo, Japan). Bacteria were precultured in Luria-Bertani (LB) containing ampicillin at 37 °C overnight, and then inoculated into M9YG medium[3] containing ampicillin and incubated at 30 °C until the OD_{600} reached to 0.5, when induction of the transformant was started by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). All other chemicals used were of analytical grade.

Synthesis of substrate analogs. NorFPP was synthesized as reported previously.[16] The synthesis of *Z*-norGGPP was started from commercially available farnesol (*E*,*E*-farnesol). Farnesol (1.5 g, 6.75 mmol) was chlorinated with *N*-chlorosuccinimide (NCS) and dimethyl sulfide (DMS) to give 1-chloro-3,7,11-trimethyl-2*E*,6*E*10-dodecatriene (1.61 g, 99.1% yield). The chloro group was displaced with ethyl cyanoacetate under lithium hydride in N,N-dimethylformamide to afford ethyl 2-cyano-5,9,13-trimethyl-4*E*,8*E*,12-tetradecatrienoate (790 mg, 37.3% yield). This ester was hydrolyzed to give 2-cyano-5,9,13-trimethyl-4*E*,8*E*-12-tetradecatrienoic acid (700 mg, 97.2% yield), which was decarboxylated to generate 2-cyano-4,8,12-trimethyl 3*E*,7*E*,12-tridecatriene (390 mg, 73.6% yield). The nitrile was reduced with diisobutylalminium hydride (DIBAL) to give 5,9,13-trimethyl-4*E*,8*E*,12-tetradecatrien-1-al (370 mg, 93.7% yield). This aldehyde was converted to a mixture of *E*- and *Z*-unsaturated esters with high selectivity (*cis*:*trans* = 10:1) using a new Horner-Emmons reagent.[22] The *cis* and *trans* mixtures were separated on silica gel flash column chromatography to give a *cis* ester, ethyl 7,11,15-trimethyl-2*Z*,6*E*,10*E*,14-hexadecatetraenoate (150 mg, 21.3% yield). The *cis* ester was reduced with DIBAL to give 7,11,15-trimethyl-2*Z*,6*E*,10*E*,14-hexadecatetraen-1-ol (120 mg, 54.5% yield). After chlorination of this alcohol with NCS and DMS (100 mg, 78.1% yield), diphosphorylation of the chloro derivative was carried out according to the method of Davisson *et al.*[20] (20.0 mg, 12.1% yield). The total yield was 0.61%. At each step, analytical TLC was performed to check the reaction product on precoated silica gel plates 60F-254, purchased from Merck. IR, ^{1}H-NMR, and ^{13}C-NMR characterized each chemical reaction product. For example, 7,11,15-trimethyl-2*Z*,6*E*,10*E*,14-hexadecatetraen-1-ol: IR (cm^{-1}) 3332, 1028; ^{1}H-NMR ($CDCl_3$) δ 1.65 (12H, s), 2.07 (12H, dt), 4.18 (2H, d), 5.11 (3H, t), 5.59 (2H, dt); ^{13}C-NMR ($CDCl_3$) δ 16.0, 17.6, 23.3, 25.6, 26.4, 26.5, 27.6, 27.8, 31.9, 39.8, 58.5, 123.4, 123.9, 124.3, 124.8, 128.6, 132.6, 135.9, 136.1. Purification was performed by silica gel flash column chromatography except for the final step of the procedure. The diphosphate produced was finally purified by reversed phase column chromatography.

Preparation of UPP synthase. UPP synthase was overproduced in *E. coli* BL21 (DE3) cells with pET22bMLU, the expression plasmid encoding *M. luteus* B-P 26 UPP synthase.[3]

BL21(DE3)/pET22bMLU were precultured in Luria-Bertani (LB) containing ampicillin at 37 °C overnight, and then innoculated into a 250-fold volume of M9YG medium containing ampicillin. The cells were grown at 37 °C to an approximate OD_{600} value of 0.3, IPTG was added to a final concentration of 1 mM, and then incubation was continued for additional 5 h at 30 °C. The enzymes were purified according to the purification procedure as described in our previous work, which includes three chromatographic steps. The fractions of the enzyme were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie Brilliant Blue staining, and fractions that showed more than 90% purity were used in

further characterization. Protein concentrations were measured by the method of Bradford, with bovine serum albumin as the standard.[23)]

UPP synthase assay. Enzyme activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. The incubation mixture for UPP synthase standard assay contained, in a final volume of 0.2 ml, 100 mM Tris–HCl buffer, pH 7.5, 0.5 mM $MgCl_2$, 10 μM FPP or *Z*-GGPP, 10 μM [1-^{14}C]IPP (37 MBq/mol), 0.05% (w/v) Triton X-100, and a suitable amount of purified UPP synthase. After incubation at 37 °C in a water bath for 15 min, the enzyme reaction was terminated by chilling the mixture with an ice bath. Then the products were immediately extracted with 1-butanol saturated with water, and the radioactivity in the butanol extract was measured with an Aloka LSC-1000 liquid scintillation counter (Aloka, Tokyo, Japan).

Inhibitory effect of substrate analogs. The 0.2 ml incubation mixture contained varying concentrations of inhibitor in addition to the contents for the standard assay. For steady-state kinetic studies, the concentration of allylic substrate FPP/*Z*-GGPP was varied, while the other substrate [1-^{14}C]IPP was kept constant at 200 μM. Calculation of kinetic parameters and Lineweaver-Burk plots were performed using EnzymeKinetics software version 1.5 (Trinity Software, Campton, NH).

Results and Discussion

X-ray crystallographic study of *M. luteus* B-P 26 UPP synthase[4)] has revealed that a structural P-loop motif is located in front of the large hydrophobic cleft, and a sulfate ion was found binding to the structural P-loop.[24)] Ammonium sulfate acts as a competitive inhibitor for the binding of FPP in the UPP synthase reaction, suggesting that the structural P-loop motif is consistent with the allylic substrate binding site.[4,18)]

In order to elucidate further the manner of allylic substrate intermediate binding, allylic substrate analogs norFPP and *Z*-norGGPP, which lack the methyl group at the 3-position of FPP and *Z*-GGPP respectively, were synthesized and examined for UPP synthase activity.

Neither norFPP nor *Z*-norGGPP showed any reactivity as a substrate for the UPP synthase reaction. The methyl group at the 3-position of the allylic substrate is strictly essential in order for *cis*-type prenyl diphosphate synthase to start the prenyltransferase reaction by eliminating the diphosphate group to generate the allylic cation.

The inhibitory effects of norFPP and *Z*-norGGPP on the UPP synthase reaction were examined. Lineweaver-Burk plot analysis of steady-state kinetic experiments with varying substrate and inhibitor concentrations showed that norFPP is a competitive inhibitor of FPP with a K_i value of 45 μM. But with respect to *Z*-GGPP,

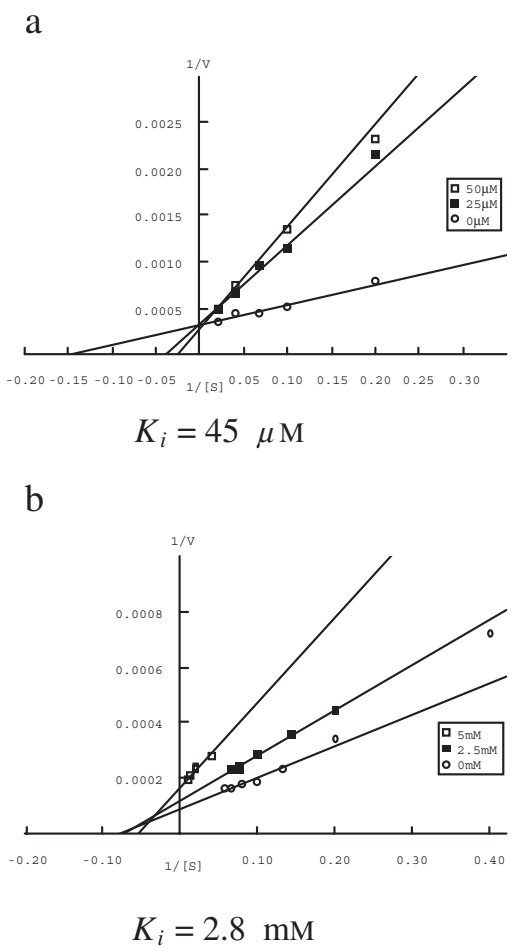

Fig. 2. Lineweaver-Burk Plots of norFPP with Respect to FPP (a) and to *Z*-GGPP (b).

These plots were obtained at three different inhibitor concentrations on UPP synthase. a, 0 (○), 25 (■), and 50 μM (□); b, 0 (○), 2.5 (■), and 5.0 mM (□).

norFPP was found to be a mixed inhibitor, and the K_i value was 60-fold larger than that of FPP, 2.8 mM (Fig. 2). These results suggest that the hydrophobic cleft for the recognition site of the hydrocarbon moiety of the intermediate *Z*-GGPP and those of longer ones having *cis*-prenyl units formed by further chain elongation is different from that of the natural allylic primer substrate, FPP. The mixed manner of inhibition implies that part of the recognition site of the diphosphate moiety of the allylic substrate is common to FPP and *Z*-GGPP.

The K_i value for *Z*-norGGPP was 17 μM with respect to FPP and 28 μM with respect to *Z*-GGPP. Lineweaver-Burk plot analysis showed mixed inhibition with respect to FPP, and a competitive manner with respect to *Z*-GGPP (Fig. 3). These results suggest that *Z*-norGGPP competes with the binding site common to *Z*-GGPP and probably to the longer *cis*-prenyl diphosphate intermediates.

Site-directed mutagenesis experiments and crystal structure analysis have revealed several amino acid

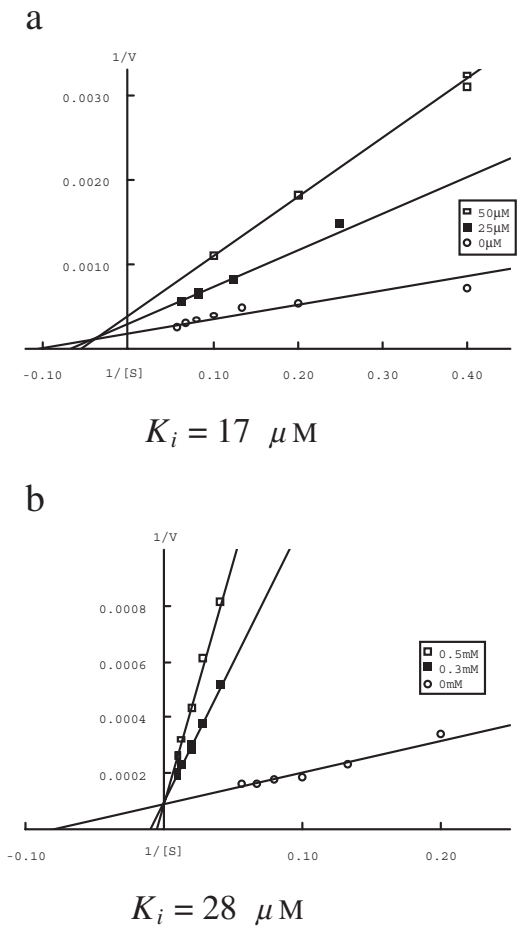

Fig. 3. Lineweaver-Burk Plots of *Z*-norGGPP with Respect to FPP (a) and to *Z*-GGPP (b).

These plots were obtained at three different inhibitor concentrations on UPP synthase. a, 0 (○), 25 (■), and 50 μM (□); b, 0 (○), 0.3 (■), and 0.5 mM (□).

residues important for FPP recognition.[19,25] These residues might be involved only in recognition of the hydrocarbon moiety of FPP, not in that of *Z*-GGPP and longer *cis*-prenyl diphosphate intermediates. For example, a mutant enzyme, R33A, showing substitution of Arg-33 for Ala, showed greatly increased K_m values for FPP and *E*-GGPP, with 30-fold decreased k_{cat} values, indicating that Arg-33 in the structural P-loop is important in the binding of FPP, but the K_m values of R33A for *Z*-GGPP, the first intermediate during the enzymatic *cis*-condensations of IPP onto FPP, showed comparable values to that of the wild-type enzyme. These results suggest that the binding features of the two allylic substrates, FPP and *Z*-GGPP, are distinctly different with each other.[18]

We proposed a hypothetical allylic substrate-binding model in our previous work.[18] According to this model, hydrocarbon and diphosphate moieties of newly formed *cis*-prenyl diphosphate binding sites were different from those of FPP. Later, the several crystal structures of *E. coli* UPP synthase in complex of substrates or substrate analogs were determined. These structures have revealed dynamic conformational changes between the crystal structure of UPP synthase with and without allylic substrates.[25–29] A conformational change was observed in the large hydrophobic cleft. The structural P-loop motif, involving recognition of the diphosphate moiety of FPP, was not different, even in a crystal structure with two Triton X-100 molecules mimicking the hydrocarbon moiety of UPP with a suitable torsion angle in the hydrophobic cleft.[6]

Kharel *et al.* found that a mutant which contained an insertion of five charged or polar amino acid residues in the helix-3 region of the large hydrophobic cavity of the UPP synthase changed the catalytic activity.[30] The wild-type enzyme produces prenyl products with carbon chain lengths of C_{55-70}, but the mutant with the insertion of five Ala residues instead did not produce the longer prenyl products.[25] These results and an analysis of crystal structure complex with Triton X-100[6] suggest that certain factors, probably charged amino acid residues in the hydrophobic cleft, control the growing and binding of the hydrophobic prenyl chain of allylic substrate intermediates.

Further elucidation of binding manner as to the allylic substrate intermediate, site-directed mutagenesis, and crystal structure determination with these 3-desmethyl allylic substrate analogs are required for a more detailed understanding of the substrate-binding sites of UPP synthase.

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