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Kinetic studies on the prenyl chain elongation by undecaprenyl diphosphate synthase with artificial substrate homologues

S. Ohnuma, T. Koyama and K. Ogura

Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai 980, Japan

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In the undecaprenyl diphosphate synthase reaction, an allylic substrate homologue, (2*Z*,6*E*,10*E*)-4-methyl-geranylgeranyl diphosphate was found to be a potent competitive inhibitor against the allylic primer, (2*Z*,6*E*,10*E*)-geranylgeranyl diphosphate. On the other hand, it acted as a strong noncompetitive inhibitor against isopentenyl diphosphate. On the basis of these facts, the topology of the substrate-binding sites as well as the reason why the synthase reaction with (*E*)-3-methyl-3-pentenyl diphosphate always stops completely at the first stage of condensation, yielding an allylic diphosphate with a methyl group at the 4-position, are discussed.

Undecaprenyl diphosphate synthase; Prenyltransferase; Prenyl chain elongation; Polyprenyl diphosphate; Artificial substrate; Substrate homologue

1. INTRODUCTION

Undecaprenyl diphosphate synthase (EC 2.5.1.31) catalyzes the consecutive condensation of eight molecules of isopentenyl diphosphate (IPP) with farnesyl diphosphate (FPP) as the priming substrate to afford the C_{55}-prenyl diphosphate, required as a carbohydrate carrier in the biosynthesis of bacterial cell envelope components. On studying the substrate specificity of this enzyme with respect to artificial substrate homologues, we recently found that (*E*)-3-methyl-3-pentenyl diphosphate (EHIPP) acted as a homoallylic substrate in the reaction with several allylic diphosphates [1,2]. Although the synthase reaction with EHIPP proceeded in the same stereochemical manner as that with the natural homoallylic substrate, IPP [3], it resulted in a full-stop at the stage where a single condensation of the C_6-homologue with an allylic primer is completed to form a chiral prenyl diphosphate with an extra methyl group at the 4-position. No allylic diphosphate with an extra methyl group at the 4-position was accepted as substrate by this enzyme even when IPP was the homoallylic substrate [2]. This paper discusses the topology of the binding sites of undecaprenyl diphosphate synthase on the basis of kinetic studies with these artificial substrate homologues.

Correspondence address: K. Ogura, Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai 980, Japan

Abbreviations: IPP, isopentenyl diphosphate; FPP, (all-*E*)-farnesyl diphosphate; (2*Z*)-GGPP, (2*Z*,6*E*,10*E*)-geranylgeranyl diphosphate; 4-MeGGPP, (*RS*)-(2*Z*,6*E*,10*E*)-4-methyl-geranylgeranyl diphosphate; EHIPP, (*E*)-3-methyl-3-pentenyl diphosphate; ZHIPP, (*Z*)-3-methyl-3-pentenyl diphosphate; PP_i, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane

2. MATERIALS AND METHODS

[1-^{14}C]IPP was purchased from Amersham. FPP, (2*Z*)-GGPP, 4-MeGGPP, EHIPP, and ZHIPP were the same preparations as used previously [1,2]. Undecaprenyl diphosphate synthase was partially purified from *Bacillus subtilis* cells according to the procedure of Takahashi et al. [4], and it was confirmed that the synthase fraction was free of any other prenyltransferases.

2.1. *Assay of undecaprenyl diphosphate synthase*

The standard incubation mixture contained, in a final volume of 1.0 ml, 50 μmol of Tris-HCl buffer, pH 8.5, 250 nmol of $MgCl_2$, 2.5 mg of Triton X-100, 5 μmol of 2-mercaptoethanol, 25 μmol of NH_4Cl, 5 μmol of KF, 25 nmol of [1-^{14}C]IPP (spec. act. 1 Ci/mol), 25 nmol of FPP or (2*Z*)-GGPP, and an appropriate amount of undecaprenyl diphosphate synthase. The reaction mixture was incubated at 37°C for 2 h, and the reaction was terminated by chilling the mixture with an ice bath. The mixture was extracted with 3 ml of butanol and an aliquot of the organic layer was counted for radioactivity. The enzymatic activity was measured by determining the amount of [^{14}C]IPP incorporated into butanol-extractable polyprenyl products as described previously [2]. One unit of the enzyme activity represents 1 nmol of IPP incorporated into product per min.

In the kinetic experiments with artificial substrate homologues, the 1-ml incubations contained varying concentrations of substrates and inhibitors in addition to the contents for the standard assay. Double reciprocal plots were delineated by the least-squares method.

3. RESULTS

The inhibitory effect of substrate analogues on the undecaprenyl diphosphate synthase reaction was studied. As shown in fig.1, the homoallylic

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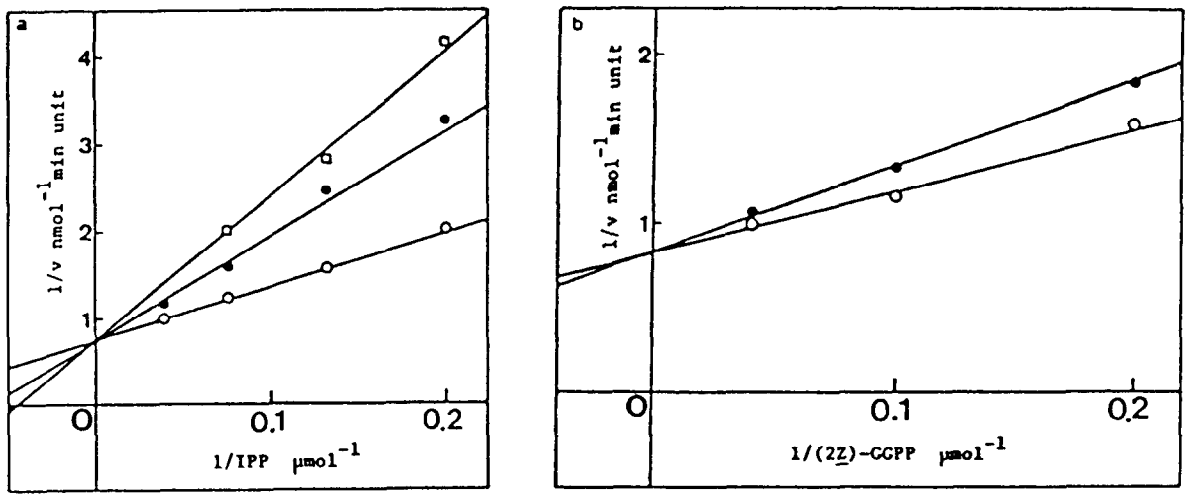


Fig.1. Inhibition of undecaprenyl diphosphate synthase with EHIPP. (a) The concentration of IPP was varied and that of (2*Z*)-GGPP kept constant at 25 μM. Concentrations of EHIPP: (○) none; (●) 50 μM; (□) 100 μM. (b) The concentration of (2*Z*)-GGPP was varied and that of IPP kept constant at 25 μM. Concentrations of EHIPP: (○) none; (●) 25 μM.

diphosphate EHIPP, which has been found to be reactive as substrate [1], showed typical competitive inhibition profiles in the double-reciprocal plots with respect to either IPP or (2*Z*)-GGPP. The inhibition constants, K_i values for EHIPP were calculated to be 75 μM and 41 μM against (2*Z*)-GGPP and IPP, respectively. The *Z*-isomer ZHIPP, which was not accepted as substrate by the enzyme [1], exhibited no inhibition on the synthase reaction.

The allylic homologue 4-MeGGPP, which has been shown to be inactive as substrate at all, was a potent competitive inhibitor against (2*Z*)-GGPP with a K_i value of 5.4 μM (fig.2). This value is comparable to the K_m value for (2*Z*)-GGPP (8.9 μM). On the other hand, 4-MeGGPP acted as a noncompetitive inhibitor against IPP with a K_i value of 5.8 μM, which was also comparable to the K_m value for IPP (8.5 μM). (*RS*)-(all-*E*)-4-Methylfarnesyl diphosphate also showed competitive inhibition against (2*Z*)-GGPP with a K_i value of 8.0 μM.

4. DISCUSSION

The undecaprenyl diphosphate synthase reaction with EHIPP proceeds in the same stereochemical manner as that with the natural substrate IPP, but the prenyl chain elongation stops fully at the first stage of condensation, forming (*S*)-4-MeGGPP [1]. Moreover, 4-MeGGPP is not accepted as substrate by this enzyme even when IPP is the counterpart substrate [2]. The

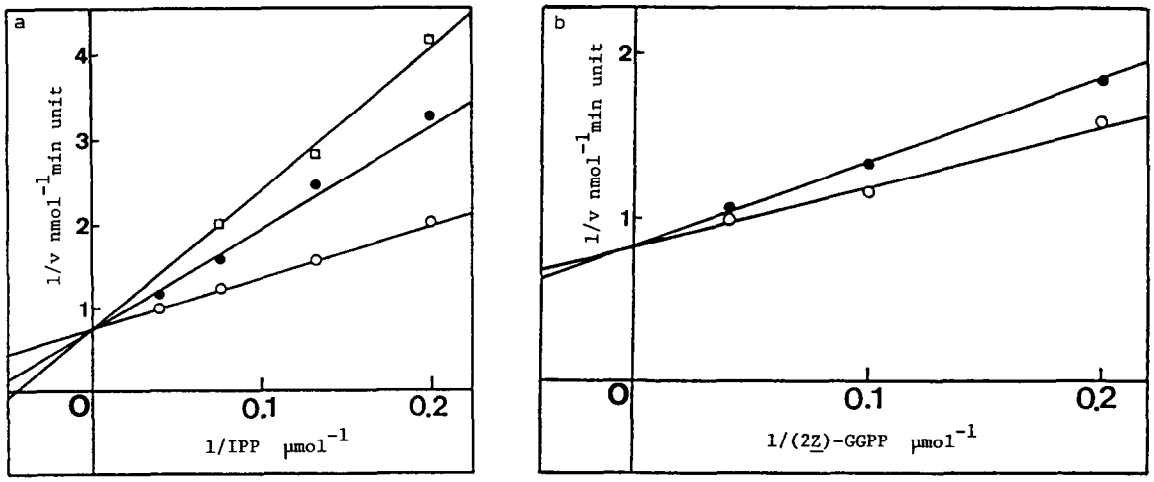
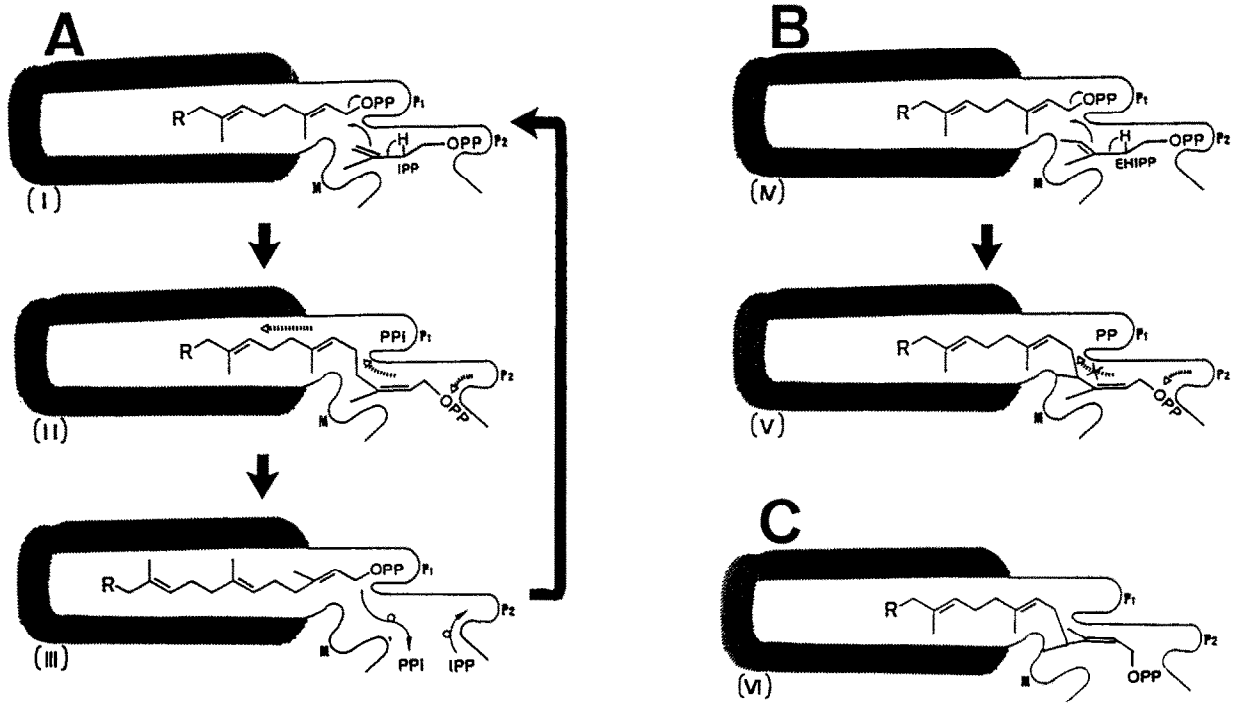


Fig.2. Inhibition of undecaprenyl diphosphate synthase with 4-MeGGPP. (a) The concentration of IPP was varied and that of (2*Z*)-GGPP kept constant at 25 μM. (b) The concentration of (2*Z*)-GGPP was varied and that of IPP kept constant at 25 μM. In both experiments the concentrations of 4-MeGGPP were: (○) none; (●) 25 μM; (□) 50 μM.

present study shows that 4-MeGGPP is a potent competitive inhibitor against (2Z)-GGPP, whereas it acts as a noncompetitive inhibitor against IPP. These facts indicate that undecaprenyl diphosphate synthase has two distinct binding sites, one for the allylic substrates and the other for IPP, like farnesyl diphosphate synthase [5]. They also suggest that 4-MeGGPP binds to the allylic-binding site competitively with the natural allylic substrates but not suitably enough to react with IPP and that the IPP binding site is not hindered by the binding of 4-MeGGPP. Once the enzymatic condensation between EHIPP and an allylic diphosphate is completed the product, which has a methyl group at the 4-position, should migrate and fit the allylic-binding site to condense with a second molecule of EHIPP if the chain elongation reaction proceeds without stopping at the first stage of condensation. The full-stop of the reaction of EHIPP therefore suggests that the extra methyl group at the 4-position prevents this migration for steric reasons. Thus, the products resulting from the reactions of EHIPP with any allylic diphosphates remain in the active site without undergoing further reaction with IPP.

Assuming that the allylic substrate binds to the enzyme first as indicated for FPP synthase reaction [6,7], we propose a possible mechanism of undecaprenyl diphosphate synthase reaction as follows (scheme 1). In the reaction with natural substrates (A), an allylic diphosphate binds to the allylic-binding site by recognizing both of the sites for the diphosphate moiety (P_1) and the hydrophobic moiety (hatched area). Then, IPP binds to the IPP site by recognizing both of the diphosphate site (P_2) and the methyl site (M) and undergoes the condensation (I). Immediately after the condensation, PP_i still remains in the P_1 site (II). Then the PP_i is displaced by the diphosphate moiety of the newly synthesized allylic diphosphate, which migrates from the IPP-binding site, leaving it open to another IPP (II⟶III). The allylic diphosphate binds to the proper position of the allylic-binding site and condenses with IPP. Similarly the reaction continues until the prenyl chain length reaches C_{55}. In the reaction with EHIPP (B-IV), the C-C bond formation occurs in the same stereochemical manner as that with the natural substrate IPP [4]. The product (*S*)-4-MeGGPP, however, cannot bind to the proper position in the allylic-binding site probably because of steric hindrance (V). Hence, the undecaprenyl diphosphate synthase reaction with EHIPP always stops at the stage where a single condensation is completed irrespective of the chain length of the counterpart allylic diphosphate. For similar topological reasons, no allylic diphosphate with an extra methyl group at the 4-position can bind to the exact position of the allylic-binding site (C), but it competitively and noncompetitively inhibits the binding of natural allylic substrates and IPP, respectively, by binding to the hydrophobic region of the allylic-binding site (VI).



Scheme 1. A mechanism of undecaprenyl diphosphate synthase reaction: (A) reaction with natural substrates, FPP and IPP; (B) reaction with FPP and EHIPP; (C) possible binding of an allylic diphosphate that has an extra methyl group at the 4-position.

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