Purification of Solanesyl-diphosphate Synthase from 
Micrococcus luteus

A NEW CLASS OF PRENYLTRANSFERASE*

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The activity of solanesyl-diphosphate synthase from Micrococcus luteus is stimulated by a high molecular mass fraction (HMF) which is separated from cell-free extracts of the same bacterium by DEAE-Toyopearl chromatography followed by Sephadex G-100 chromatography. By employing HMF in the assay procedure, solanesyl-diphosphate synthase was able to be purified to homogeneity and was found to be a homodimer with a monomeric molecular mass of 34 kDa. In contrast to hexaprenyl- and heptaprenyl-diphosphate synthases, which are composed of two easily dissociable components that are inactive unless combined, the homogeneously purified solanesyl-diphosphate synthase itself showed a catalytic activity, though weak, catalyzing the synthesis of both (all-E)-nonaprenyl-(solanesyl-)(all-E-nonaprenyl)-(solanesyl-) and (all-E)-octaprenyl diphosphate. HMF does not affect the stability of solanesyl-diphosphate synthase or $K_v$ values for isopentenyl diphosphate and farnesyl diphosphate, but it markedly increases $V_{max}$ values in a time-dependent manner. Several lines of evidence indicate that HMF contains a factor which binds to polypropenyl products and removes them out of the active site of enzyme to facilitate and maintain the turnover of catalysis.

Prenyltransferases, which are responsible for the fundamental chain elongation in isoprenoid biosynthesis, can be classified into several groups in terms of the mode of requirement for catalytic activity. Geranyl- (1, 2), farnesyl- (3–7), and geranylglyceranyl- (8–12) diphosphate synthases require for their activities no cofactors except divalent metal ions such as Mg$^{2+}$ or Mn$^{2+}$, which are commonly required by all prenyltransferases. The prenyltransferases that catalyze the formation of (Z)-polyprenyl chains including undecaprenyl- (9, 13–18), nonaprenyl- (19), and dehydrodolichyl- (20, 21) diphosphate synthases require phospholipid or detergent for their catalytic activities. On the other hand, hexaprenyl-diphosphate synthase (22, 23) from Micrococcus luteus B-P 26 and heptaprenyl-diphosphate synthase (24, 25) from Bacillus subtilis, which catalyze the formation of (E)-polyprenyl chains, are unique because they do not require lipid or detergent but comprise two easily dissociable protein components, neither of which has any catalytic activity. It has been thought that the unique properties of these enzymes can account for their abilities to catalyze the synthesis of water-insoluble material from soluble substrates without aid of detergent molecules (28).

By analogy, it was expected that prenyltransferases responsible for the synthesis of (E)-polyprenyl diphosphates with chain lengths longer than C$_{30}$ would have properties similar to those of hexaprenyl- and heptaprenyl-diphosphate synthases. This seemed likely in view of the fact that solanesyl (all-E-nonaprenyl)-diphosphate synthase (SPP synthase) partially purified from M. luteus, unlike undecaprenyl-diphosphate synthase, does not require phospholipid or detergent. It has also been known that the partially purified enzyme catalyzes the synthesis of all-E-C$_{40}$-prenyl diphosphate as well as all-E-C$_{40}$-prenyl diphosphate under normal conditions (26) and that the chain lengths of products vary within the longest length of C$_{40}$ depending on the concentration of Mg$^{2+}$ ions in the reaction (27). However, it remained unclear whether the variability of the product chain length is a nature of a single enzyme, because further purification of the enzyme was difficult since the specific activity was apt to decrease during the course of purification.

The present studies were undertaken in order to clarify this point by purifying the enzyme to homogeneity as well as to learn whether the unique two-dissociable component system is common to all-E-polyprenyl-diphosphate synthases. This paper describes the achievement of purification of SPP synthase and the finding that this enzyme is quite different from hexaprenyl- and heptaprenyl-diphosphate synthases in terms of the constitution of subunits and the mode of activation.

MATERIALS AND METHODS

Reagents—[1-$^3$H]Isopentenyl diphosphate (IPP) (2.035 TBq/mol) was purchased from Amersham Corp. Nonlabeled IPP and (E,E)-farnesyl diphosphate (FFP) were synthesized according to the procedures of Davisson et al. (29). DEAE-Toyopearl 650M and butyl-Toyopearl 650M were purchased from Tosoh Co., Ltd. Sephadex G-100, Mono Q, and Superose 12 were purchased from Pharmacia LKB Biotechnology Inc. Cells of M. luteus were purchased from Seikagaku Kogyo Co., Ltd. Lysozyme, deoxyribonuclease, bovine serum albumin, and acid phosphatase were purchased from Boehringer Mannheim. (all-E)-Geranylglyceranol was a generous gift from Kuray Co., Ltd. (all-E)-Decaprenol and solanesol were kindly donated from Nishin Flour Milling Co., Ltd. (2Z,6Z,10Z,14Z,18Z,22E,26E)-Octaprenol was extracted and purified from Betula schmidtii. Precast reversed phase thin layer chromatography plates (LKC-18) were purchased.
from Whatman Chemical Separation, Inc. All other chemicals were of analytical grade.

**SPP Synthase Assay**—The enzyme activity was assayed as usual by measuring the amount of incorporation of [1-14C]IPP into butanol-extractable polypropenyl diphosphates. In a standard experiment the assay mixture contained, in a final volume of 1.0 ml, 20 μmol of Tris-HCl buffer (pH 7.7), 5 μmol of MgCl₂, 25 nmol of FPP, 25 nmol of [1-14C]IPP (37 GBq/mol), SPP synthase, and HMF, the preparations of which are described later. The mixture was incubated at 37 °C for 1 h, and the reaction was stopped by chilling the reaction mixture in an ice bath. The mixture was shaken with 3.0 ml of butanol that had been saturated with H₂O. The butanol phase was back-washed with water, and the radioactivity in the butanol phase was determined.

**Analysis of Products**—The polypropenyl diphosphates produced by the enzymatic reactions were treated with acid phosphatase according to the method of Fuji et al. (30). The hydrolysates were extracted with pentane, and the pentane-soluble products were analyzed by reversed phase LKC-18 thin layer chromatography and silica gel chromatography developed with a solvent system of acetone/H₂O (19:1) and benzene/ethyl acetate (9:1), respectively. The positions of authentic standards were visualized with iodine vapor. Distribution of radioactivity was detected by scanning the plate with an Aloka radiochromatogram scanner.

**Preparation of HMF and Purification of SPP Synthase**—Freeze-dried cells (30 g) were suspended in 450 ml of 50 mM Tris-HCl buffer (pH 7.7) containing 1 mM EDTA (buffer A). Lysozyme (150 mg) was added to the cell suspension, and the mixture was stirred for 40 min at room temperature. The lysate was then treated with deoxyribonuclease (30 mg) at 0 °C for 1 h and centrifuged at 100,000 g for 1 h. The supernatant fraction was chromatographed on a DEAE-Toyopearl 650M column (7 × 25 cm) equilibrated with buffer A. Elution was performed with a gradient of 0–850 mM NaCl (Fig. 1). The SPP synthase fractions (fraction numbers 102–110) were pooled and concentrated by ultrafiltration with a YM 10 membrane (Amicon Co. Ltd.). The concentrated enzyme solution was applied to a Sephadex G-100 column (4 × 72 cm) equilibrated with buffer A. Elution was performed with buffer A (see Fig. 6A). The SPP synthase activity was stimulated by fraction I (fraction numbers 27–37), which is designated as HMF. The SPP synthase activity was found in fraction II (fraction numbers 39–46) with a reduced yield (see Fig. 6B). The enzyme fraction was chromatographed on a Mono Q column (10 × 100 mm) equilibrated with buffer A. Elution was performed with a programmed gradient of 0–400 mM NaCl (Fig. 2). The SPP synthase fractions (retention time, 27–39 min) were combined and then chromatographed on a Butyl-Toyopearl 650M column (1.6 × 10 cm) equilibrated with buffer A containing ammonium sulfate at 40% saturation. Elution was performed with a decreasing linear gradient of 0–400 mM NaCl (Fig. 3). The SPP synthase fractions (retention time, 37–39 min) were combined and purified by DEAE-Toyopearl 650M chromatography (Fig. 4).

![Fig. 1. DEAE-Toyopearl 650M chromatography of the 100,000 x g supernatant fraction.](image)

![Fig. 2. Mono Q chromatography of the Sephadex G-100 purified SPP synthase.](image)

![Fig. 3. Butyl-Toyopearl 650M chromatography of the Mono Q-purified SPP synthase.](image)
THE PURIFICATION OF SPP SYNTHASE

The purification procedure of SPP synthase included two kinds of gel filtration. As shown in Fig. 6A, a high molecular mass fraction (HMF) of SPP synthase was isolated after Superose 12 chromatography. The DEAE-Toyopearl-purified enzyme was chromatographed on a Sephadex G-100 column (7.5%). The assay methods were described under "Materials and Methods." The assay for enzyme activity was carried out with a supplement of HMF. The native PAGE-purified SPP synthase gave on SDS-PAGE a single protein band corresponding to a molecular mass of 34 kDa. The enzyme seems to be a dimer with a monomeric molecular mass of 34 kDa. The pure SPP synthase was catalytically active even in the absence of HMF, but it was activated by HMF more markedly than was a partially purified preparation of SPP synthase.

RESULTS

Preparation of HMF—When the DEAE-Toyopearl-purified SPP synthase (Fig. 1) was chromatographed on a Sephadex G-100 column (Fig. 6A), the enzymatic activity decreased to less than 10% of that before the chromatography. It has been shown that some bacterial prenyltransferases have multi-component systems; hexaprenyl-diphosphate synthase (22, 23) from M. luteus B-P 26 or heptaprenyl-diphosphate synthase (24, 25) from B. subtilis is composed of two easily dissociable and nonidentical subunits. On the other hand, undecaprenyl-diphosphate synthase, which is composed of non-dissociable and identical subunits, requires some lipids for its catalytic activity. Recently, rubber synthase (33-36) from Hevea brasiliensis has been found to be composed of two nonidentical subunits, one having a FPP synthase activity and the other an elongation factor. Assuming that a factor or factors which affect the activity of SPP synthase were deprived during the Sephadex G-100 chromatography, we first searched for such a factor in the fractions obtained in the gel filtration. As shown in Fig. 6B, a high molecular mass fraction (1, fraction numbers 27-37) stimulated the SPP synthase activity. This fraction, designated as HMF, was used in the following experiments.

Purification of SPP Synthase—The purification procedure of M. luteus SPP synthase included two kinds of gel filtrations, two kinds of ion exchange chromatographies, hydrophobic chromatography and native PAGE (Table I). The assay for enzyme activity was carried out with a supplement of HMF. The native PAGE-purified SPP synthase gave on SDS-PAGE a single protein band corresponding to a molecular mass of 34 kDa (Fig. 7). Since the molecular mass of SPP synthase was 74-78 kDa as estimated by either Superose 12 (Fig. 4) or Sephadex G-100 chromatography (Fig. 6A), the enzyme seems to be a dimer with a monomeric molecular mass of 34 kDa. The pure SPP synthase was catalytically active even in the absence of HMF, but it was activated by HMF more markedly than was a partially purified preparation of SPP synthase.

Product Analysis—The products formed by the reaction of the native PAGE-purified SPP synthase in the presence or absence of HMF were analyzed. As the reaction products were diphosphate esters, they were hydrolyzed with acid phosphatase as usual (30) and the hydrolysates were analyzed by means of reversed phase and silica gel thin layer chromatographies. As shown in Fig. 8, in the case of the reaction of pure SPP synthase without addition of HMF, one of the major radioactive products migrated to the region where authentic (all-E)-nonaprenol (solanesol) migrated. Another radioactive product migrated faster than authentic solanesol and slower than authentic (Z,6Z,10Z,14Z,18Z,22E,26E)-octaprenol. In view of the fact that an (all-E)-polypropenyl migrates slower than its Z,E-mixed isomer (24), these products seem to be (all-E)-nonaprenol (Cα) and (all-E)-octaprenol (Cα). These products co-migrated with the authentic Cα and CαI prenols on silica gel thin layer chromatography respectively (data not shown). The distribution of these products is similar to that observed with partially purified SPP synthase (26). The distribution pattern of the products formed in the absence of HMF was almost the same as that of the products formed in its presence.

High Molecular Fraction Which Activates SPP Synthase—HMF was found to be precipitable with ammonium sulfate at 50% saturation (data not shown). In order to see whether or not lipids are involved in HMF, we carried out lipid extraction experiments according to the Bligh-Dyer method (37). As shown in Table II, the lipid extracts did not stimulate the activity of SPP synthase, but the water-soluble fraction did stimulate the enzyme activity, although the effect was slightly lower than that of the untreated HMF. Anthrone reagent was used for detection of any saccharides in HMF, but no saccha-
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TABLE I

Purification of M. luteus SPP synthase

|        | Total Protein | Total Activity | Specific Activity | Purification | Recovery |
|--------|---------------|----------------|-------------------|--------------|----------|
| mg     | nmol/min      | nmol/min/mg    | -fold             | %            |
| Crude lysate* | 100,000 x g supernatant* | 1289 | 67.0 | 0.052 | 1.0 | 100 |
| DEAE-Toyopearl | 6.8 | 27.9 | 0.13 | 2.5 | 41 |
| Sephadex G-100 | 0.28 | 11.6 | 41 | 803 | 17 |
| Mono Q | 219 | 67.0 | 0.052 | 1.0 | 100 |
| Butyl-Toyopearl | 0.093 | 6.8 | 73 | 1408 | 10 |
| Native PAGE | ND | ND | ND | ND | ND |

* Precise amounts of SPP synthase could not be determined by the assay method described under “Materials and Methods,” because these fractions contained other prenyltransferases (farnesyl-, geranylgeranyl-, and unde-caprenyl-diphosphate synthases) and phosphatase, which would interfere in the measurement of SPP synthase activity.

** ND, not determined.

SDS-PAGE

Fig. 7. SDS-PAGE of the native PAGE-purified SPP synthase. The native PAGE-purified SPP synthase was electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS and silver-stained. Lane 1, molecular weight markers; lane 2, native PAGE-purified SPP synthase.

rides were detected. By assaying the enzymatic activity using HMF instead of FPP, we also ascertained that HMF did not contain any endogenous allylic diphosphates that would act as primer substrates. From these facts, the stimulation seems to be attributable to protein in HMF.

The effect of HMF on SPP synthase activity is shown in Fig. 9A. As the amount of HMF was increased, the enzyme activity increased up to approximately 3-fold. Bovine serum albumin (BSA) also showed a similar stimulative effect (Fig. 9B).

SPP synthase itself was labile against heat treatment, but HMF was stable even when treated at 55 °C for 10 min (Fig. 10A). We therefore examined whether or not the activation by HMF is a result of stabilization of SPP synthase against heat treatment. The addition of HMF, however, did not stabilize the enzyme (Fig. 10B).

The amount of the products formed by the enzyme reaction was determined as a function of incubation period in the presence or absence of HMF (Fig. 11A). In these experiments, 40 μg of the Mono Q-purified SPP synthase and 750 μg of HMF protein were employed. In the presence of HMF, the reaction proceeded almost linearly for 4 h. In its absence, however, the reaction reached a plateau within 1 h. Namely,

The lipid fraction and the water-soluble fraction were prepared from HMF according to the method of Bligh and Dyer (38). Solvents of these fractions were evaporated in vacuo, and the residues were dissolved in water. The activity was assayed under the standard conditions described under “Materials and Methods” except that the lipid fraction and the water-soluble fraction were added as indicated.

| Additive                | Radioactivity | dpm |
|------------------------|---------------|-----|
| HMF*                   | 22,680        |     |
| Lipid fraction*        | 6,681         |     |
| Water-soluble fraction*| 17,441        |     |
| Lipid fraction* + water soluble fraction* | 18,026 |     |
| None                   | 3,078         |     |

* 400 μg of protein was used.

* The amount of the lipid fraction was adjusted to a level similar to that of the untreated HMF fraction.

Fig. 8. Reversed phase thin layer radiochromatograms of the alcohols formed by acid phosphatase treatment of the products of SPP synthase reaction. The reaction mixture contained, in a final volume of 0.1 ml, 2 μmol of Tris-HCl buffer (pH 7.7), 0.5 μmol of MgCl₂, 2.5 nmol of FPP, 5.5 nmol of [1-14C]IPP (2.072 TBq/mol), and the pure SPP synthase in the absence (A) or presence (B) of HMF. The mixture was incubated at 37 °C for 1 h, and the products were analyzed as described under “Materials and Methods.” The arrows indicate the migration positions of reference polyprenols: a, (all-E)-farnesol; b, (all-E)-geranylgeraniol; c, (22,62,102,142,182,22E,26E)-octaprenol (C₄₈); d, (all-E)-nonaprenol (solanesol) (C₄₅); e, (all-E)-decaprenol (C₅₀).

TABLE II

Effect of the lipid fraction and water-soluble fraction separated from HMF

The lipid fraction and the water-soluble fraction were prepared from HMF according to the method of Bligh and Dyer (38). Solvents of these fractions were evaporated in vacuo, and the residues were dissolved in water. The activity was assayed under the standard conditions described under “Materials and Methods” except that the lipid fraction and the water-soluble fraction were added as indicated.

| Additive                | Radioactivity | dpm |
|------------------------|---------------|-----|
| HMF*                   | 22,600        |     |
| Lipid fraction*        | 6,601         |     |
| Water-soluble fraction*| 17,441        |     |
| Lipid fraction* + water soluble fraction* | 18,026 |     |
| None                   | 5,078         |     |

* 400 μg of protein was used.

* The amount of the lipid fraction was adjusted to a level similar to that of the untreated HMF fraction.
the observed stimulation of the enzyme activity depended markedly on the incubation period; the activations were about 3- and 10-fold after 1 and 4 h, respectively. To examine whether the enzyme was denatured after the incubation, we carried out experiments as follows. After the enzymatic reaction was carried out in the absence of HMF for indicated periods, HMF was added to the reaction mixture, and then the mixture was incubated again at 37 °C for 1 h (Fig. 11B). The enzyme was still active, and the reaction proceeded again.

Kinetic parameters of SPP synthase reaction in the presence or absence of HMF

The reaction mixture was incubated at 37 °C for 20 min. In the absence of HMF, the reaction proceeded for this period with the rate of reaction declining. In the presence of HMF, the reaction proceeded for this period with the rate of reaction declining. However, the $K_m$ values estimated in the absence of HMF were both similar to those in its presence. Thus, HMF seems to stimulate the SPP synthase activity by increasing the initial rate of the reaction as well as maintaining the turnover of reaction for several hours.

It has been reported that the long chain polyprenyl diphosphates produced by a partially purified enzyme from *M. luteus*...
are eluted in the \( V_0 \) region of Sephadex G-25 (38). In order to examine whether HMF shows some specific interaction with the substrates or products, we carried out binding experiments by means of Superose 12 chromatography, using a mixture of \(^{14}C\)octaprenyl diphosphate and \(^{14}C\)solanesyl diphosphate synthesized from \([1-{^1}C]\)IPP and FPP by SPP synthase in the presence or absence of HMF. When the mixture resulting from the reaction carried out without HMF was chromatographed on a Superose 12 column, two peaks of radioactivity emerged (Fig. 12A). Peak II, which emerged in the \( V_t \) region, corresponded to recovered IPP, while Peak I, which emerged near the elution point of SPP synthase, corresponded to the products including solanesyl and octaprenyl diphosphates. In the case of the reaction mixture in the presence of HMF, two peaks of radioactivity also emerged near the elution point of SPP synthase, corresponding to the products including solanesyl and octaprenyl diphosphates. In the case of the reaction mixture in the presence of HMF, two peaks of radioactivity also emerged from the column (Fig. 12B). Peak II had an elution volume similar to that observed in the former column. The reaction products, solanesyl and octaprenyl diphosphates, emerged together with HMF at the \( V_0 \) region, forming Peak III. Both \([{^{1}}C]\)IPP and \([{^{1}}C]\)FPP were eluted in the \( V_t \) region of the column without regard to the presence or absence of HMF (data not shown).

In order to investigate the state of the polyprenyl products in Peak III, similar binding experiments were carried out employing BSA or bacitracin instead of HMF. It has been reported that the former binds to some hydrophobic molecules nonspecifically and that the latter exhibits high affinity for polyenyl diphosphate (39). Both BSA (Fig. 9B) and bacitracin (data not shown) also stimulated SPP synthase activity, and the extent of the stimulation depended on their concentration. When a reaction mixture containing BSA was incubated and then chromatographed on a Superose 12 column, two radioactivity peaks, Peaks IV and II, emerged from the column (Fig. 12C). The elution volume of Peak II was similar again to that observed in Fig. 12, A and B while Peak IV, which was proved to contain the enzymatic products, was eluted together with BSA. In the presence of bacitracin, the reaction mixture gave only a single peak of radioactivity in the \( V_t \) region of Superose 12 chromatography (data not shown), and it was found that the fraction corresponding to this peak contained \([{^{14}}C]\)IPP as well as the enzymatic products \([{^{14}}C]\)SPP, \([{^{14}}C]\)octaprenyl diphosphate, and bacitracin. These results indicate that not only HMF but also materials that can bind to the polyprenyl products stimulate the SPP synthase activity.

**DISCUSSION**

The discovery of the HMF, which markedly stimulates SPP synthase activity, facilitated the purification of SPP synthase from *M. luteus*, and the enzyme was purified to homogeneity. To our knowledge, this is the first report of purification to our knowledge, this is the first report of purification of SPP synthase, which catalyzes cis-chain elongation to produce \( Z,E \)-mixed \( C_{55} \)-prenyl diphosphate, essentially requires phospholipids or some detergent for its catalytic activity (13–18). Hexaprenyl-diphosphate synthase (22,23) from *M. luteus* B-P 26 or heptaprenyl-diphosphate synthase (24, 25) from B. 

![Figure 12](image-url)  
**Fig. 12.** Superose 12 chromatography of \([{^{14}}C]\)SPP and \([{^{14}}C]\) octaprenyl diphosphate produced in the SPP synthase reaction mixture. The reaction mixture contained, in a final volume of 200 ml, 4 \( \mu \)mol of Tris-HCl buffer (pH 7.7), 1 \( \mu \)mol of MgCl\(_2\), 5 \( \mu \)mol of FPP, 5 \( \mu \)mol of \([{1-{^{14}}C}]\)IPP (2.035 TBq/mol), 21 \( \mu \)g of the Mono Q-purified SPP synthase, and an activator (HMF or BSA). The mixture was incubated at 37 °C for 1 h, and then chromatographed directly on a Superose 12 column (10 \( \times \) 300 mm). The radioactivity of the eluent was measured by liquid scintillation counting. A, without the activator; B, 150 \( \mu \)g of HMF; C, 51 \( \mu \)g of BSA. ●, radioactivity in the eluent; - - - , absorbance at 280 nm.

subtilis* is composed of two dissimilar subunits, each of which has no catalytic activity at all. Recently, rubber synthase (33–36) from *H. brasiliensis* has been shown to have two different prenyltransferase activities. In the presence of "rubber elongation factor" it catalyzes the addition of multiple cis-isoprene units to rubber molecules, while in the absence of rubber elongation factor this enzyme shows FPP synthase activity, that is, the addition of two trans-isoprene units to dimethylallyl diphosphate.
On the other hand, the homogeneously purified protein in this study shows SPP synthase activity by itself. Investigation of the enzyme for its increase in specific activity during its purification led us to the finding that SPP synthase activity was stimulated by HMF. The stimulation depends on the concentration of HMF, but it is not an absolute requirement for catalytic activity. In contrast to rubber synthase, the function of which is dramatically changed by rubber elongation factor, the function of SPP synthase was not altered by HMF at all. The chain length distribution of the products of the purified SPP synthase was found to change dependent on Mg^{2+} concentration (data not shown) as observed previously (27) with a partially purified enzyme.

The fact that HMF is heat-stable is reminiscent of component A of hexaprenyl-diphosphate synthase (22) or component I of heptaprenyl-diphosphate synthase (25), but HMF cannot substitute for any of these components (data not shown). HMF does not affect the heat stability of SPP synthase in the absence or presence of substrates (Figs. 5B and 6B).

Many carrier proteins or binding proteins which directly participate in the bioconversion of hydrophobic molecules are known. Some bind to substrates and others to products. For example, sterol carrier protein-2 (43, 44) participates in the conversion of squalene to lanosterol by liver microsomal enzyme, and fatty acid-binding protein (45) exhibits a high affinity for fatty acids and their CoA esters and may participate in their intracellular transport. These proteins share a functional similarity by allowing insoluble molecules to move through and interact with components in the aqueous environment.

HMF not only increases the velocity of SPP synthase reaction depending on its concentration but also extends the linearity of the reaction. In the case of FPP synthase reaction, deprivation of the product FPP, which is a potent inhibitor (46), has been reported to be the rate-limiting step (47). As the long prenyl chain of the product of a polypropenyl-diphosphate synthase would have much hydrophobic interaction with the active site of the enzyme, it should have a mechanism to deprive the enzyme of its product to maintain the turnover of catalysis. It is reasonable to assume that HMF contains a factor that plays a functional role in removal of hydrophobic products from the active site of enzyme.

Several lines of evidence indicated that HMF contains a factor which binds to polypropenyl diphosphate synthases synthesized by SPP synthase, but not to either of the substrates, IPP and FPP. It was also shown that both BSA and bacitracin could bind to SPP and stimulate the catalytic activity of SPP synthase. The former is known to bind to some hydrophobic molecules nonspecifically, and the latter to exhibit high affinity for polypropenyl diphosphates (39). These results suggest that the factor (HMF) stimulates the enzyme activity by removing SPP from the enzyme. Trypsin treatment of HMF did not result in a complete loss of activity (data not shown), but this may indicate that the lysoform of this factor is also active rather than that the factor is not protein.

In order to see whether the stimulation effect of HMF is specific for SPP synthase from \emph{M. luteus}, we examined the effect of HMF on other prenyltransferases from various organisms. As a result, HMF did not stimulate any of undecaprenyl-diphosphate synthase from \emph{B. subtilis}, hexaprenyl-diphosphate synthase from \emph{M. luteus} B-P 26, and FPP synthase from pig liver. However, it stimulated the octaprenyl-diphosphate synthase from \emph{Escherichia coli} K-12 and decaprenyl-diphosphate synthase from \emph{Paracoccus denitrificans} (data not shown). Therefore HMF is commonly effective to bacterial prenyltransferases that catalyze the synthesis of all-E-polyprenyl diphosphates with chain lengths of C_{40}, C_{50}, and C_{50}. These three all-E-polyprenyl-diphosphate synthases, also share a similar property in that they are stimulated by Tween 80, which does not affect hexaprenyl- or heptaprenyl-diphosphate synthase (data not shown). Therefore, they should be assigned to a new class different from that for hexaprenyl- and heptaprenyl-diphosphate synthases. The involvement of HMF must be essential because of the molecular properties of these particular products that each consist of a polypropenyl chain of C_{40}, C_{50}, or C_{50} length and a diphosphate moiety.

It is surprising that the properties of \emph{M. luteus} SPP synthase reaction are quite different from those of hexaprenyl-diphosphate synthase from \emph{M. luteus} B-P 26. Although \emph{M. luteus} B-P 26 has been classified in the same \emph{Micrococcus} subgroup as the strain of ATCC 48 (48), the characteristics of these two strains are quite different from each other in that the former is nonpigmented and is resistant to lysozyme treatment.

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