A novel prenyltransferase from *Paracoccus denitrificans*

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A new polyprenyltransferase catalysing the formation of Z-double bonds was found and partially purified from extracts of *Paracoccus denitrificans*. The enzyme catalysed a consecutive condensation of isopentenyl diphosphate with *EE*-farnesyl diphosphate as a primer to produce *EE*-farnesyl-all-Z-hexaprenyl diphosphate (*ZE*-mixed nonaprenyl diphosphate) as the final product. Not only *EE*-farnesyl diphosphate but also neryl diphosphate, *ZE*-farnesyl diphosphate, *ZEE*-geranylgeranyl diphosphate and *ZZEE*-pentaprenyl diphosphate were all accepted as substrates. This polyprenyltransferase required detergent such as Triton X-100 for its catalytic activity. The formation of *ZE*-mixed undecaprenyl diphosphate, which is well known as the precursor of the bacterial sugar-carrier lipid, was not detected in extracts of this bacterium.

INTRODUCTION

Prenyltransferases catalyse the head-to-tail condensation between isopentenyl diphosphate and an allylic diphosphate to produce prenyl diphosphates with various chain lengths and stereochemistry. The enzymes so far isolated from several bacteria are classified into three groups. One of them includes short-chain prenyltransferases, which synthesize geranyl (C_{10}) diphosphate, farnesyl (C_{15}) diphosphate and geranylgeranyl (C_{20}) diphosphate (Kandutsch *et al.*, 1964; Sagami *et al.*, 1978; Takahashi & Ogura, 1981). These short-chain products are employed as priming substrates for further chain-elongation reaction by the other two groups of prenyltransferases, *E*-polyprenyltransferases and *Z*-polyprenyltransferases. In accordance with various chain lengths of menaquinone side chains, three distinct *E*-polyprenyltransferases have been isolated. They are hexaprenyl (C_{30}) diphosphate synthase (Fujii *et al.*, 1982*a*), heptaprenyl (C_{35}) diphosphate synthase (Takahashi *et al.*, 1980) and nonaprenyl (solanesyl, C_{45}) diphosphate synthase (Sagami *et al.*, 1977). As to the *Z*-polyprenyltransferase, however, only undecaprenyl diphosphate synthase has been obtained from bacteria. This is in good accordance with the fact that *ZE*-mixed-undecaprenyl (C_{55}) diphosphate, the sugar-carrier lipid for cell-wall biosynthesis, is the only *Z*-prenyl compound found in bacteria (Allen *et al.*, 1976; Baba & Allen, 1980; Takahashi & Ogura, 1982).

Recently we have studied the chain-elongation system of isoprenoid biosynthesis in *Paracoccus denitrificans*, which resembles a mitochondrion more closely than any other bacteria in that it effectively assembles the features of the mitochondrial respiratory chain and oxidative phosphorylation (Imai *et al.*, 1968). As a result, farnesyl diphosphate synthase, *E*-decaprenyl diphosphate synthase (Ishii *et al.*, 1985) and a *Z*-polyprenyltransferase were separated. Unexpectedly, however, the *Z*-polyprenyltransferase produced a *ZE*-nonaprenyl diphosphate but not the *ZE*-undecaprenyl diphosphate compound. The present paper reports the isolation and characterization of this new prenyltransferase.

EXPERIMENTAL

Materials

Cells of *Paracoccus denitrificans* were kindly donated by Niigata Research Laboratory of Mitsubishi Gas Chemical Co. (Niigata, Japan). [$1-^{14}C$]isopentenyl diphosphate and (2*S*)-[2- 3H]isopentenyl diphosphate (specific radioactivities 1.0 Ci/mol and 1.8 Ci/mmol respectively) and allylic diphosphates were the same preparations as used previously (Sagami *et al.*, 1977). Polyprenols used as reference compounds were those obtained from *Betula schmidtii* (Kurokawa *et al.*, 1971).

Enzyme assay

The enzyme activity was measured as described previously (Fujii *et al.*, 1982*a*) by determination of the amount of [$1-^{14}C$]isopentenyl diphosphate incorporated into butan-1-ol-extractable materials. In a standard experiment, the incubation mixture contained, in a final volume of 1.0 ml, 50 mM-potassium phosphate buffer, pH 8.5, 5 mM-MgCl₂, 0.5% (w/v) Triton X-100, 25 μ M-allylicdiphosphate, 25 μ M-[$1-^{14}C$]isopentenyl diphosphate and a suitable amount of enzyme protein. After incubation at 37 °C for 1 h, the reaction products were immediately extracted with butan-1-ol. The butanol layer was washed with water and a portion of the extract was taken for counting of the radioactivity. The enzyme activity was expressed as the radioactivity in the butanol extract.

Product analysis

Polyprenyl products extracted with butan-1-ol were chromatographed on a silica-gel plate with propan-1-ol/conc. NH₃/water (6:3:1, by vol.) (solvent I). Enzymic hydrolysis of the products was carried out with potato acid phosphatase (Fujii *et al.*, 1982*b*). The hydrolysates were analysed by t.l.c. and radio-h.p.l.c. The t.l.c. was carried out on a silica-gel plate with benzene/ethyl acetate (9:1, v/v) (solvent II), and radio-h.p.l.c. was performed with a 50 cm $\times$ 2.1 cm column of Hitachi porous polymer gel 3011 connected to a Berthold type LB503 radioactivity monitor. The elution of polyprenols was carried out with

methanol/hexane (4:1, v/v) at a flow rate of 0.8 ml/min, and was monitored by recording both the absorption at 216 nm and the radioactivity.

Purification of polyprenyltransferase

Washed cells of *P. denitrificans* (29 g wet wt.) were suspended in 50 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol and 1 mM-EDTA and disrupted by sonication twice for 1 min with a 2 min interval, in an ice bath, with a Branson Sonifier at maximum output. After treatment with deoxyribonuclease, the suspension was centrifuged at 95 500 g for 30 min. Subsequent steps were carried out at 0–4 °C. The protein fraction precipitated from the supernatant by 30–60% saturation with $(\text{NH}_4)_2\text{SO}_4$ was dialysed and chromatographed on a hydroxyapatite column (38 cm $\times$ 2.7 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol, 1 mM-MgCl₂ and 20% (w/v) glycerol. Elution was performed with a linear concentration gradient of 10–300 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol (total volume 1400 ml). The fractions having polyprenyltransferase activity were combined and subjected to hydroxyapatite chromatography repeated twice in the same manner.

RESULTS

When a 30–60% -satn. $(\text{NH}_4)_2\text{SO}_4$ fraction obtained from *P. denitrificans* was chromatographed on hydroxyapatite with phosphate buffer containing 20% glycerol, and assayed with [$1\text{-}^{14}\text{C}$]isopentenyl diphosphate and dimethylallyl diphosphate, a single prenyltransferase activity peak corresponding to farnesyl diphosphate synthase (fraction A) was observed (Fig. 1). Although the findings are not shown here, fraction A also contained all-*E*-decaprenyl diphosphate synthase. Another prenyltransferase (B), which was active with *ZEE*-geranylgeranyl diphosphate as substrate, was eluted over a broad range, overlapping partially with fraction A. Rechromatography of fraction B on hydroxyapatite gave a polyprenyltransferase fraction free of farnesyl diphosphate synthase and *E*-decaprenyl diphosphate synthase (Fig. 2).

The reaction mixture resulting from an incubation of [$1\text{-}^{14}\text{C}$]isopentenyl diphosphate and farnesyl diphosphate with the hydroxyapatite-purified enzyme was extracted with butan-1-ol, and the extract was subjected to silica-gel t.l.c. in solvent I. Since a single radioactivity peak (R_F 0.36) appeared between all-*E*-geranylgeranyl diphosphate (R_F 0.31) and its monophosphate (R_F 0.45), the reaction product was thought to be a diphosphate ester having a chain length longer than C_{20} . The radiochromatogram of the silica-gel t.l.c. of the hydrolysate obtained by acid phosphatase treatment of the butanol extract showed a broad radioactivity peak over the R_F range 0.4–0.6, whereas the R_F values of *ZEE*-geranylgeraniol and *EE*-farnesyl-all-*Z*-farnesol, *EE*-farnesyl-all-*Z*-geranylgeraniol, *EE*-farnesyl-all-*Z*-pentaprenol and all-*E*-geranylgeranyl-all-*Z*-heptaprenol were 0.44, 0.50, 0.53, 0.55 and 0.63 respectively. However, h.p.l.c. resolved the same hydrolysate into two major and four minor components. The radio-h.p.l.c. showed radioactivity peaks at retention times of 4.7, 5.6, 7.0, 8.1, 11.0 and 14.4 min with relative intensities of 1.00, 0.58, 0.03, 0.05, 0.08 and 0.14 respectively. The retention times of 4.7, 7.0, 8.1 and 11.0 min coincided with those of authentic specimens of

Fig. 1. Chromatography of a 30–60% -satn. $(\text{NH}_4)_2\text{SO}_4$ fraction on hydroxyapatite

Enzyme activity was assayed as described in the Experimental section. ○, Enzyme activity assayed with [$1\text{-}^{14}\text{C}$]isopentenyl diphosphate and dimethylallyl diphosphate; △, enzyme activity assayed with [$1\text{-}^{14}\text{C}$]isopentenyl diphosphate and *ZEE*-geranylgeranyl diphosphate; ----, A_{280} . The arrow indicates the starting point of gradient elution.

Fig. 2. Hydroxyapatite chromatography of fraction B

Enzyme activity was assayed with as allylic substrates dimethylallyl diphosphate (○), geranyl diphosphate (□) and *ZEE*-geranylgeranyl diphosphate (△). ----, A_{280} .

Table 1. Stereospecific incorporation of isopentenyl diphosphate into prenols

	³ H (d.p.m.)	¹⁴ C (d.p.m.)	³ H/ ¹⁴ C ratio
[1- ¹⁴ C, (2 <i>S</i>)-2- ³ H]isopentenyl diphosphate	124836	156226	0.80
<i>ZE</i> -mixed-Octaprenol* (C ₄₀)	0	30090	0.00
<i>ZE</i> -mixed-Nonaprenol (C ₄₅)	0	24011	0.00
<i>EE</i> -Farnesol†	33732	41486	0.81
all- <i>E</i> -Decaprenol (C ₅₀)	26227	31812	0.82

* The incubation mixture contained, in a final volume of 2.0 ml, the same components as described in the Experimental section except that [1-¹⁴C]isopentenyl diphosphate was replaced by [1-¹⁴C, (2*S*)-2-³H]isopentenyl diphosphate. *EE*-Farnesyl diphosphate was the allylic substrate. The *ZE*-mixed-polyprenols obtained by phosphatase treatment were purified by h.p.l.c. as described in the Experimental section and their radioactivities counted.

† For the reference experiments, the condensation of double-labelled isopentenyl diphosphate with dimethylallyl diphosphate and with *EE*-farnesyl diphosphate were performed by farnesyl diphosphate synthase and *E*-decaprenyl diphosphate synthase purified from *P. denitrificans* respectively. All-*E*-Prenols were obtained essentially by the same methods as described above, and their radioactivities counted.

ZEE-geranylgeraniol, *EE*-farnesyl-all-*Z*-farnesol, *EE*-farnesyl-all-*Z*-geranylgeraniol and *EE*-farnesyl-all-*Z*-pentaprenol.

For further analysis, the major polyprenol fractions obtained by a large-scale incubation were purified by silica-gel t.l.c. followed by h.p.l.c., and they were subjected to mass-spectrometric analysis. These poly-prenols exhibited typical fragmentation patterns of isoprenoid alcohols, showing molecular ions at *m/z* 562 and *m/z* 630 corresponding to octaprenol (C₄₀H₈₆O) and nonaprenol (C₄₅H₉₄O) respectively. These results, taken in conjunction with the results from studies on the stereochemical course of reaction and the substrate specificity as described below, clearly indicate that the present prenyltransferase catalyses condensation between isopentenyl diphosphate and *EE*-farnesyl diphosphate to give *ZE*-mixed polyprenyl diphosphate consisting of nine isoprene units as the longest prenyl product.

The stereochemical course of this polyprenyltransferase reaction was compared with those in the reactions of farnesyl diphosphate synthase and *E*-decaprenyl diphosphate synthase separated from this bacterium, with [1-¹⁴C, (2*S*)-2-³H]isopentenyl diphosphate and a suitable co-substrate. It was shown that the 2-pro-*S*-hydrogen atom of isopentenyl diphosphate was exclusively lost during the reaction of this enzyme, whereas the same hydrogen atom was retained during the reactions of both farnesyl diphosphate synthase and *E*-decaprenyl diphosphate synthase (Table 1).

The substrate specificity of the polyprenyltransferase was examined, by using various geometric isomers of C₁₀, C₁₅, C₂₀ and C₂₅ prenyl diphosphates (Table 2). Both *ZE*- and *EE*-isomers of farnesyl diphosphate were active as substrates, the latter being more reactive than the former, whereas the isomers having a *Z*-double bond in the α-residue of C₁₀, C₂₀ and C₂₅ prenyl groups were better substrates than the corresponding all-*E*-isomers. No

Table 2. Reactivities of the polyprenyltransferase towards allylic substrates

The incubations were carried out as described in the Experimental section. The concentration of each allylic diphosphate was 50 μM. Abbreviation: -PP, diphosphate.

Substrate	Enzyme activity (d.p.m.)
Dimethylallyl-PP	0
Geranyl-PP	1200
Neryl-PP	2684
<i>EE</i> -Farnesyl-PP	4140
<i>ZE</i> -Farnesyl-PP	3280
<i>EEE</i> -Geranylgeranyl-PP	3284
<i>ZEE</i> -Geranylgeranyl-PP	4303
<i>EEEE</i> -Pentaprenyl-PP	1524
<i>EZEE</i> -Pentaprenyl-PP	446
<i>ZEEE</i> -Pentaprenyl-PP	1977
<i>ZZEE</i> -Pentaprenyl-PP	3368

Fig. 3. Effect of detergents on the enzyme activity

The enzyme activity was measured with *EE*-farnesyl diphosphate under the standard conditions except that detergent was varied as indicated. The detergents used were Triton X-100 (○), Tween 80 (●), sodium dodecyl sulphate (□) and sodium deoxycholate (■).

polyprenyl product longer than C₄₅ was synthesized from any of the substrates.

The enzyme required detergent for its catalytic activity. As shown in Fig. 3, Triton X-100 was the most effective, and the enzyme activity was enhanced with increasing concentration of this detergent up to 0.05% (w/v). Phosphatidylglycerol and lipid extracts from *P. denitrificans* cells also activated the enzyme, the optimum concentrations both being 0.05%. In the presence of 0.05% of phosphatidylglycerol and 0.05% of the lipid

Fig. 4. Radio-h.p.l.c. of polyprenols liberated by the treatment of enzyme reaction products with acid phosphatase

The enzymic reactions were carried out in the presence of 0.2 mM- (a), 1.0 mM- (b) and 5.0 mM-MgCl₂ (c) with *EE*-farnesyl diphosphate as the allylic substrate.

extracts the enzyme respectively showed activities of 0.58 and 0.30 relative to the maximum activity in the presence of Triton X-100. The enzyme was not stimulated by univalent cations such as K⁺, Na⁺ and NH₄⁺.

When 0.01% of Triton X-100 was used, *ZE*-mixed-nonaprenyl diphosphate was exclusively synthesized from isopentenyl diphosphate and *EE*-farnesyl diphosphate by the hydroxyapatite-purified enzyme. Similarly, when a crude extract from this bacterium was used as enzyme with *ZEE*-geranylgeranyl diphosphate as the primer without any detergent, the major product was also the C₄₅ compound. The product distribution was also dependent on the concentration of Mg²⁺ in the incubation mixture. The amount of C₄₅ product relative to products shorter than C₄₅ increased as Mg²⁺ concentration was elevated. However, the chain length never exceeded C₄₅ even at 5 mM-Mg²⁺ (Fig. 4).

DISCUSSION

The prenyltransferase reported here catalyses the oligomerization of isopentenyl diphosphate with *EE*-farnesyl diphosphate as a primer, forming *Z*-double bonds to give *ZE*-mixed-nonaprenyl diphosphate as the final product. Therefore we propose to call this enzyme *Z*-nonaprenyl diphosphate synthase as a trivial name.

In bacteria so far investigated, undecaprenyl phosphate is well known as the glycosyl-carrier lipid in the biosynthesis of cell-wall components (Hemming, 1981). Although attempts were made with hydroxyapatite-purified *P. denitrificans* enzyme to change the reaction conditions so that the distribution of the products might be changed, the chain length of the longest product never exceeded C₄₅. Even in the reaction with crude extracts from this bacterium as enzyme, the formation of *ZE*-mixed-undecaprenyl diphosphate was not detected in the presence or in the absence of Triton X-100. Because of the inability of the extracts of *P. denitrificans* to synthesize *ZE*-mixed-undecaprenyl diphosphate, the *ZE*-mixed-nonaprenyl diphosphate synthesized by this enzyme may be employed as a sugar-carrier lipid in the biosynthesis of cell-wall components of this bacterium.

This enzyme shows similar substrate specificity to that of undecaprenyl diphosphate synthase (Allen *et al.*, 1976; Baba & Allen, 1980; Takahashi & Ogura, 1982). The enzyme is much more active with *EE*-farnesyl diphosphate and all-*E*-geranylgeranyl diphosphate than with geranyl diphosphate, and is inactive with dimethylallyl diphosphate.

Purified *Z*-nonaprenyl diphosphate synthase, like undecaprenyl diphosphate synthase of other bacteria, requires detergent for its catalytic activity. Phosphatidylglycerol, which is the major phospholipid in *P. denitrificans* (Wilkinson *et al.*, 1972), also activates this enzyme. The present enzyme is insensitive to univalent cations, whereas the undecaprenyl diphosphate synthase of *Bacillus subtilis* (Takahashi & Ogura, 1982) has been reported to be stimulated markedly by K⁺ or NH₄⁺. It is of interest that *E*-decaprenyl diphosphate synthase separated from *P. denitrificans* is activated by detergents and univalent cations (results not shown). These facts indicate that the present enzyme may be peripherally associated with membranes in a fashion different from that of *E*-decaprenyl diphosphate synthase.

The change of product distribution depending on the concentration of Mg²⁺ in the reaction mixture has been observed in the reaction catalysed by *E*-polyprenyltransferases such as hexaprenyl diphosphate synthase (Fujii *et al.*, 1982a) and solanesyl diphosphate synthase (Fujii *et al.*, 1980). In the case of the present prenyltransferase, the product is changed depending not only on the concentration of Mg²⁺ but also on that of Triton X-100. The similar tendency observed with these three prenyltransferases may give a clue to understanding of the factors regulating the termination of the biological polymerization of isoprene units.

In any chromatography this enzyme was eluted over a broader range than were the other prenyltransferases in this bacterium in the presence or in the absence of glycerol in the eluent. Gel filtration through Sephadex G-100 resulted in the loss of more than 90% of the enzyme activity. The unusual chromatographic behaviour of this enzyme may be accounted for by assuming that there is

a factor that makes this enzyme stable and associate to form several sizes of aggregates. A lipid material may participate in this behaviour, since addition of 0.1% Triton X-100 instead of glycerol to a crude enzyme solution results in a complete loss of the enzyme activity in 2 days at 4 °C.

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