Solanesyl Pyrophosphate Synthetase from *Micrococcus lysodeikticus*†

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ABSTRACT: Solanesyl pyrophosphate synthetase from extracts of *Micrococcus lysodeikticus* was purified by DEAE-Sephadex, hydroxylapatite, and Sephadex G-100 chromatography. This enzyme was found to catalyze the trans condensation of isopentenyl pyrophosphate with geranyl pyrophosphate to afford *all-trans*-octaprenyl (C_{40}) and *all-trans*-nonaprenyl (C_{45}) pyrophosphate without accumulation of prenyl pyrophosphate with chain length shorter than C_{40}. *all-trans*-Farnesyl and *all-trans*-geranylgeranyl pyrophosphate also were active as cosubstrates, though they were less effective than geranyl pyrophosphate. However, neither dimethylallyl nor *cis,trans,trans*-geranylgeranyl pyrophosphate was active. The molecular weight of this enzyme was estimated to be 78 000 by Sephadex G-100 filtration. An enzyme preparation from young shoots of potato was found to hydrolyze the polyprenyl pyrophosphates effectively to give the corresponding prenols.

Allen et al. (1967) reported a partial purification of long-chain prenyl pyrophosphate synthetase from *M. lysodeikticus* which catalyzed the conversion of isopentenyl pyrophosphate plus allylic pyrophosphate, such as farnesyl pyrophosphate, into a mixture of polyprenyl pyrophosphate ranging in carbon chain from C_{35} to C_{50} with a predominance of C_{35} and C_{40}. Subsequently, a previous report from this laboratory showed that a crude enzyme preparation of the same bacterium catalyzed the formation of undecaprenyl phosphate as well as lower isoprenol analogues (Kurokawa et al., 1971). However, the relation between the enzymes in these two reports has remained to be clarified, and, besides, the stereochemistry of these products has not been known. It is important to establish the stereochemistry of these polyprenyl pyrophosphates to see whether they are precursors for the *all-trans*-prenyl side chain of quinones, such as vitamin K_2 or for undecaprenyl phosphate of cis, trans mixed stereochemistry which is known as carbohydrate carrier lipid in the biosynthesis of cell-wall components. It is also our interest to see whether a single enzyme catalyzes the synthesis of polyprenyl pyrophosphates with such a variety of chain length.

Therefore, we studied the structure of the products using an enzyme preparation obtained by a modification of the method of Allen et al. (1967) and demonstrated that this enzyme catalyzed specifically the formation of *all-trans*-octaprenyl and nonaprenyl pyrophosphate. This paper describes these results.

Materials and Methods

Spray-dried cells of *M. lysodeikticus* was purchased from Worthington Biochemical Co. Young shoots of potato were obtained locally. Dimethylallyl pyrophosphate, geranyl pyrophosphate, *all-trans*-farnesyl pyrophosphate, and [1-^{14}C]isopentenyl pyrophosphate (1.2 Ci/mol) were the same preparations as used in the previous studies (Shinka et al., 1975). *all-trans*-Geranylgeranyl pyrophosphate and *cis,-trans,trans*-geranylgeranyl pyrophosphate were prepared as described later. (3*R*,4*S*)-[4-^{3}H]- and (3*S*,4*R*)-[4-^{3}H]mevalonic acid (168.3 Ci/mol) and (3*R*,4*R*)-[4-^{3}H]- and (3*S*,4*S*)-[4-^{3}H]mevalonic acid (84.05 Ci/mol) were products of Amersham. The solanesol used as a reference was kindly given by Nisshin Flour Co. Avicel (microcrystalline cellulose) and hydroxylapatite were purchased from Funakoshi Chemicals Co. and Seikagaku Kogyo, Japan, respectively. Molecular weight standards, cytochrome *c*, chymotrypsinogen A, albumin, and aldolase were products of Boehringer Mannheim GmbH.

Synthesis of all-trans- and cis,trans,trans-Geranylgeranyl Pyrophosphate. A mixture of *all-trans*- and *cis,trans,trans*-geranylgeraniol obtained from geranyllinalool by the method of Bates et al. (1963) was separated on a Hitachi No. 3011 column (8 × 500 mm) with methanol at a flow rate of 2.0 mL/min with a Hitachi liquid chromatograph 635. The elution of the alcohols was monitored by recording the absorption at 215 nm. Complete separation was achieved by four times recycling. The purified alcohols were phosphorylated essentially by the method of Cramer and Böhm (1959), as modified by Kandutsch et al. (1964), and purified by silica gel thin-layer chromatography with a solvent system of 1-propanol–ammonia–water (6:3:1, v/v)(solvent I).

Synthesis of (2R)-[2-^{3}H]Isopentenyl Pyrophosphate and (2S)-[2-^{3}H]Isopentenyl Pyrophosphate. (2*R*)-[2-^{3}H]Isopentenyl pyrophosphate and (2*S*)-[2-^{3}H]isopentenyl pyrophosphate were enzymatically synthesized from (3*R*,4*S*)-[4-^{3}H]mevalonic acid (168.3 Ci/mol) and from (3*R*,4*R*)-[4-^{3}H]mevalonic acid (84.05 Ci/mol), respectively, essentially by the method of Cornforth and Popják (1969). The reaction mixture contained, in a final volume of 6.0 mL, 600 μmol of Tris-HCl[1] buffer (pH 7.4), 30 μmol of $MgCl_2$, 12 μmol of $MnCl_2$, 12 μmol of adenosine triphosphate, 12 μmol of glutathione, 48 μmol of iodoacetamide, 8.96 μCi of (3*R*,4*S*)-[4-^{3}H]- and (3*S*,4*R*)-[4-^{3}H]mevalonic acid or 8.65 μCi of (3*R*,4*R*)-[4-^{3}H]- and (3*S*,4*S*)-[4-^{3}H]mevalonic acid, and 3.84 mg of 30–50% ammonium sulfate fraction obtained from a 100 000*g* supernatant of pig liver homogenates. After 1 min of preincubation without iodoacetamide and labeled mevalonic acid, iodoacetamide was added and the mixture was incubated for 4 min, and then the complete reaction mixture was incubated at 37 °C for 10 min. The reaction mixture was subjected to paper chromatography in a solvent system of

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[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl.

tert-butyl alcohol–formic acid–water (40:10:15, v/v)(Bloch et al., 1959). Radioactive isopentenyl pyrophosphate with an R_f 0.58 was extracted from the paper with aqueous ammonia solution (0.01%, v/v). Each ^{3}H-labeled isopentenyl pyrophosphate thus obtained was mixed with [1-^{14}C]isopentenyl pyrophosphate in an appropriate ratio as described later and was used in the stereochemical experiment.

Enzyme Assay. The enzyme activity was assayed as usual for prenyltransferase by determining the amount of incorporation of [1-^{14}C]isopentenyl pyrophosphate into acid-labile allylic pyrophosphate. In the standard experiments the assay mixture contained, in a final volume of 1.0 mL, 100 μmol of Tris-HCl buffer (pH 7.4), 10 μmol of $MgCl_2$, 20 nmol of geranyl pyrophosphate, 50 nmol of [1-^{14}C]isopentenyl pyrophosphate, and enzyme. The reaction mixture was incubated at 37 °C for 3 h, during which the formation of products was almost linear with time. To the reaction mixture was added 0.2 mL of 6 N HCl and the mixture was incubated at 65 °C for 20 min. The hydrolysates were extracted with 5.0 mL of hexane after the addition of 0.4 mL of 6 N NaOH and 0.4 mL of 2 M NaCl. The radioactivity in the hexane extract was routinely determined in toluene scintillation fluid.

Thin-Layer Chromatography. Polyprenyl pyrophosphates were extracted with 1-butanol from the reaction mixture and chromatographed on silica gel sheets (Merck) in solvent I. For the separation of free prenols, two systems were employed. In one system a plate coated with silica gel was developed with benzene–ethyl acetate (9:1, v/v)(solvent II) or benzene–ethyl acetate (4:1, v/v)(solvent III). In the other system (reversed phase) a plate coated with Avicel was impregnated with paraffin oil by dipping once into a 5% solution of liquid paraffin in petroleum ether (bp 40–50 °C), and the plate was developed with acetone–water (17:3, v/v) saturated with paraffin oil (solvent IV). The distribution of radioactivity was determined by scanning the developed plate with an Aloka radiochromatoscanner. Spots of reference alcohols, *all-trans*-farnesol, *all-trans*-geranylgeraniol, geranyllinalool, and solanesol were located by exposing the plate to iodine vapor.

Preparation of Polyprenyl Pyrophosphate Synthetase. All steps were carried out at 4 °C unless otherwise stated. Polyprenyl pyrophosphate synthetase was purified by a modified procedure of Allen et al. (1967) from extracts of *M. lysodeikticus* cells obtained by their method. The protein fraction precipitating between 30 and 50% saturation of ammonium sulfate was dissolved in a minimum volume of 0.05 M Tris-HCl buffer (pH 7.4) and was filtered through Sephadex G-25 equilibrated with 0.05 M phosphate buffer (pH 6.8) containing 0.05 M NaCl. The resulting solution containing 150 mg of protein was chromatographed on a DEAE-Sephadex A-50 column (1.5 × 20 cm) equilibrated with the same buffer. The elution was carried out with a linear gradient of 0.05–1.05 M NaCl in 0.05 M phosphate buffer (pH 6.8) (total volume 500 mL). Six-gram fractions were collected and assayed for enzyme activity by the standard procedure described above. The fractions having polyprenyl pyrophosphate synthetase activities were combined. For further purification, the combined fraction was applied to a hydroxylapatite column (1.3 × 13 cm) equilibrated with 1 mM phosphate buffer (pH 6.8). The protein was eluted with a linear gradient of 1–100 mM phosphate buffer. Fractions containing 150 drops were collected and assayed for polyprenyl pyrophosphate synthetase activities. The fractions having the enzyme activity were combined. The combined fraction was concentrated to 1 mL and chromatographed on a Sephadex G-100 column (1.7 × 53 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 6.8). Fractions of 1.6 mL were collected, and the fractions (fraction numbers 29–34) having polyprenyl pyrophosphate synthetase activity were pooled. The purified enzyme was stable for at least 2 weeks when stored frozen at −20 °C. Protein concentration was estimated by measuring optical density at 280 nm.

Phosphatase Preparation. A crude phosphatase preparation capable of hydrolyzing polyprenyl pyrophosphates to the corresponding prenols was obtained from young shoots of potato. Potato shoots (9.5 g, ca. 10-cm long) developed in the dark were grated in 7.6 mL of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM β-mercaptoethanol at 4 °C. The grated mixture was filtered through gauze and the filtrate was centrifuged at 3000 rpm for 2 min. The supernatant was used as immediately as possible.

Preparation of Polyprenyl Pyrophosphates and Their Dephosphorylation. Large quantities of polyprenyl pyrophosphates required for determining their structures were prepared using an incubation mixture 100 times as large as that for the standard assay. The incubation time was extended to 20 h to maximize the product formation. The products were analyzed by two methods. In one method, polyprenyl pyrophosphates in the reaction mixture were hydrolyzed by the addition of hydrochloric acid at 65 °C for 20 min, and the hydrolysates were extracted with hexane as described above. The free polyprenyl products in the hexane extracts were subjected to silica gel thin-layer chromatography in solvent II. In the other method, polyprenyl pyrophosphates, either with or without extraction with 1-butanol from the reaction mixture, were treated with a potato phosphatase preparation obtained as described above. The reaction mixture for the hydrolysis of polyprenyl pyrophosphate contained, in a final volume of 200 mL, 100 mL of the reaction mixture containing polyprenyl pyrophosphate obtained by a large-scale incubation as described above, 40 mL of 1% (w/v) Triton X-100, and 60 mL of the phosphatase preparation. The mixture was incubated at 37 °C for 24 h. The resulting free prenols were extracted three times from the reaction mixture with ca. 200-mL portions of hexane after the addition of 40 mL of 6 N NaOH. The combined extracts were washed with water, concentrated, and subjected to silica gel thin-layer chromatography in solvent II. The radioactive polyprenols were extracted with ether from the silica gel scraped from the radioactive region of the plate and the extracts were subjected to high-speed liquid chromatography. Two radioactive polyprenols isolated were analyzed by mass spectrometry.

Results

Purification of Polyprenyl Pyrophosphate Synthetase. The enzyme fractions precipitating between 30 and 50% ammonium sulfate from the 60 000*g* supernatant of *M. lysodeikticus* extracts were chromatographed on DEAE-Sephadex A-50 with a NaCl linear gradient elution (Figure 1). The distribution of enzyme activity was determined by measuring the amount of the conversion of [1-^{14}C]isopentenyl pyrophosphate in the presence of allylic pyrophosphate into acid-labile materials. Analysis of the products showed that the first peak corresponded to geranylgeranyl pyrophosphate synthetase and the second peak to polyprenyl pyrophosphate synthetase. The first peak also contained isopentenyl pyrophosphate isomerase, since this fraction converted isopentenyl pyrophosphate into geranylgeranyl pyrophosphate even when incubated in the absence of allylic pyrophosphate. The enzyme fractions corresponding to the second peak were combined and were further purified on hydroxylapatite (Figure 2) and Sephadex G-100 (Figure 3). The molecular weight of the polyprenyl pyrophosphate synthetase was estimated to be 78 000 by Sephadex G-100 filtration.

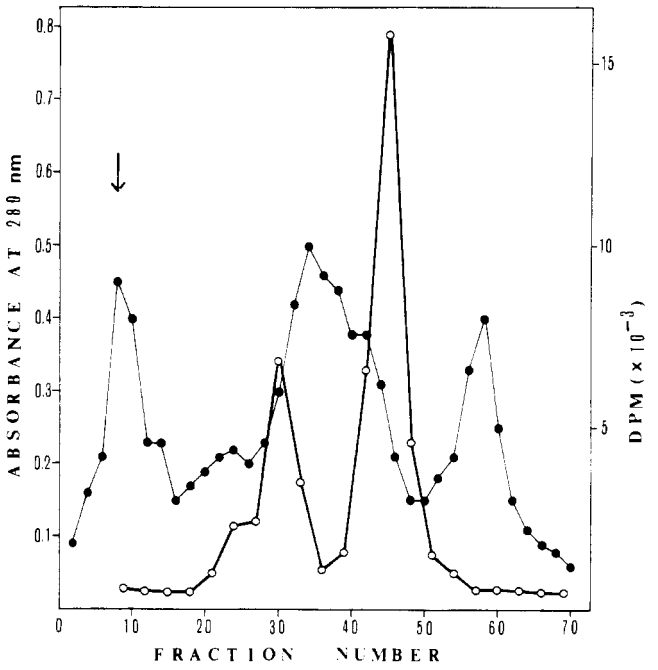

FIGURE 1: DEAE-Sephadex A-50 chromatography of a 30–50% ammonium sulfate fraction: (●–●) optical density at 280 nm; (○–○) enzyme activity when [1-^{14}C]isopentenyl pyrophosphate and geranyl pyrophosphate were the substrates. The arrow represents the starting point of the linear gradient.

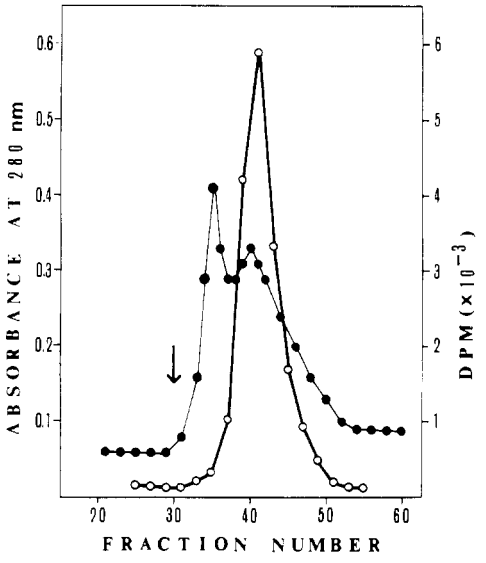

FIGURE 2: Hydroxylapatite chromatography of DEAE-Sephadex purified enzyme (corresponding to the second activity peak): (●–●) optical density at 280 nm; (○–○) enzyme activity with [1-^{14}C]isopentenyl pyrophosphate and geranyl pyrophosphate as substrates. The arrow represents the starting point of the linear gradient.

Substrate Specificity. The effectiveness of five allylic pyrophosphates as cosubstrates with [1-^{14}C]isopentenyl pyrophosphate was compared at various concentrations using Sephadex G-100 purified enzyme. As shown in Figure 4, geranyl pyrophosphate showed a conspicuous reactivity, and both *all-trans*-farnesyl pyrophosphate and *all-trans*-geranylgeranyl pyrophosphate had considerable activities. On the other hand, *cis,trans,trans*-geranylgeranyl pyrophosphate was completely inactive. These results indicate that this enzyme is not responsible for the synthesis of undecaprenyl pyrophosphate with mixed stereochemistry which is known to occur in *M. lysodeikticus*, but it catalyzes the formation of polyprenyl pyrophosphate with an all-trans configuration at least up to the tetraprenyl terminal from the ω end. It is noteworthy that the reactivity of dimethylallyl pyrophosphate is as low as negligible.

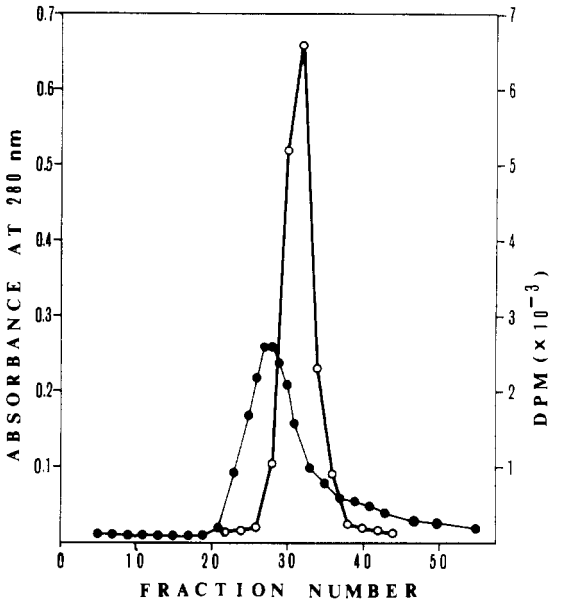

FIGURE 3: Sephadex G-100 chromatography of hydroxylapatite-purified enzyme: (●–●) optical density at 280 nm; (○–○) enzyme activity with [1-^{14}C]isopentenyl pyrophosphate and geranyl pyrophosphate as substrates.

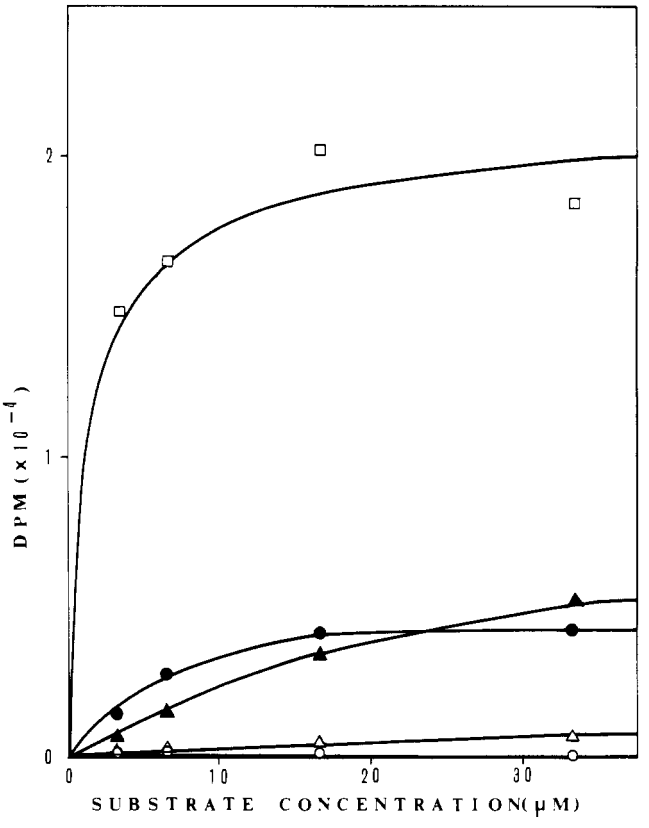

FIGURE 4: Effect of substrate concentration on the reaction velocity. The assay mixture contained, in a final volume of 1.5 mL, 100 μmol of Tris-HCl buffer (pH 7.4), 5 μmol of $MgCl_2$, 50 nmol of [1-^{14}C]isopentenyl pyrophosphate, allylic pyrophosphate, and 43 μg of Sephadex G-100 purified enzyme. Incubation time was 150 min. The allylic substrates are dimethylallyl pyrophosphate (△), geranyl pyrophosphate (□), *all-trans*-farnesyl pyrophosphate (▲), *all-trans*-geranylgeranyl pyrophosphate (●), and *cis,trans,trans*-geranylgeranyl pyrophosphate (○).

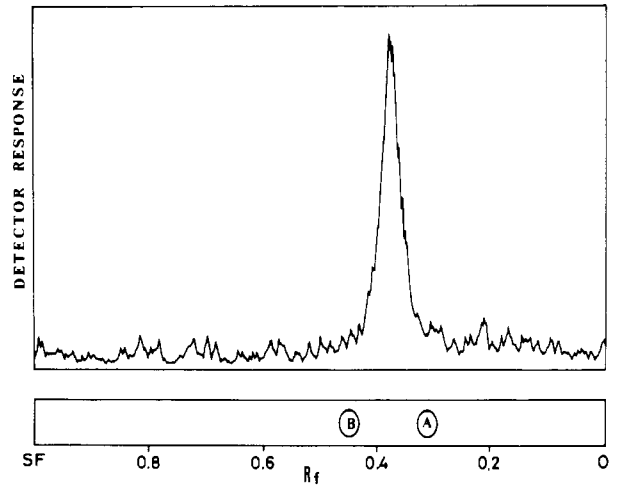

FIGURE 5: Thin-layer radiochromatogram of the 1-butanol extract of the reaction mixture of [1-^{14}C]isopentenyl pyrophosphate and geranyl pyrophosphate using DEAE-Sephadex A-50 purified enzyme. A silica gel plate was used in solvent I. Spots of reference: A, geranylgeranyl pyrophosphate; B, geranylgeranyl monophosphate.

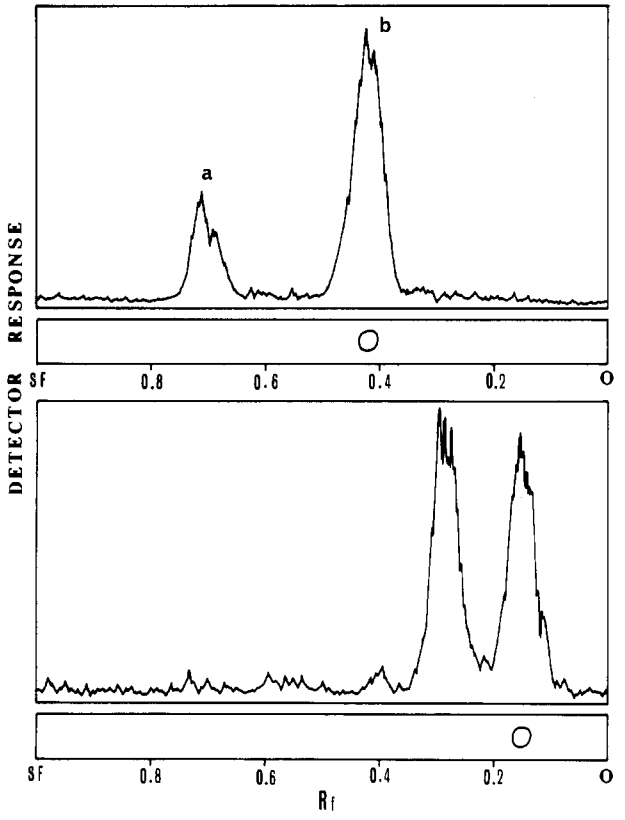

FIGURE 6: *Upper*, thin-layer radiochromatogram of hexane-soluble materials obtained by potato phosphatase treatment of the products derived from [1-^{14}C]isopentenyl pyrophosphate and geranyl pyrophosphate. A silica plate was used in solvent II. The spot indicates solanesol located with iodine vapor. *Lower*, reversed-phase thin-layer radiochromatogram of the materials obtained by ether extraction of the section corresponding to radioactivity peak b in *upper* figure. The sample was mixed with reference solanesol and chromatographed on an Avicel plate impregnated with paraffin oil in solvent IV. The spot indicates reference solanesol located with iodine vapor.

Identification of Products. The polyprenyl pyrophosphate synthesized from geranyl pyrophosphate and [1-^{14}C]isopentenyl pyrophosphate had an R_f 0.38 in silica gel thin-layer chromatography in solvent I (Figure 5), in which geranylgeranyl pyrophosphate and geranylgeranyl monophosphate

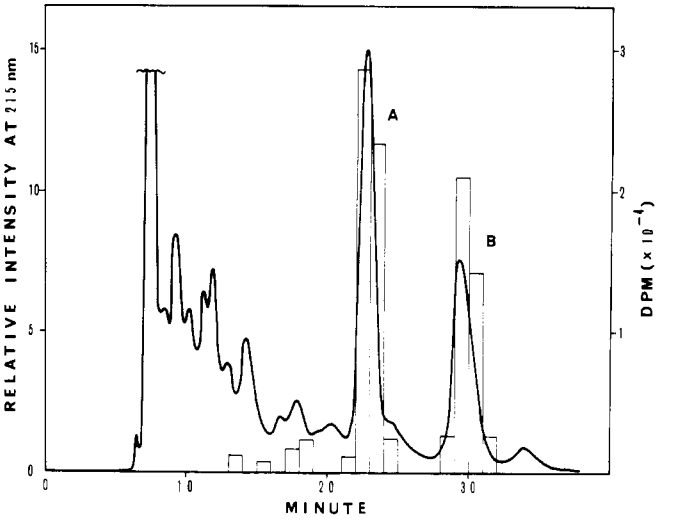

FIGURE 7: High-speed liquid chromatography of the polyprenols obtained by ether extraction of the section corresponding to radioactivity peak B in Figure 6. The elution was performed with methanol–hexane (4:1, v/v) at a flow rate 2.0 mL/min on a Hitachi No. 3011 column (8 × 500 mm) with a UV absorbance detector (at 215 nm). The histogram indicates radioactivity.

as references had R_f's 0.31 and 0.44, respectively. This indicates that the radioactive material is a pyrophosphate ester with a chain length longer than C_{20}.

Treatment of the pyrophosphate products with hydrochloric acid gave a mixture of at least three components (I, II, and III), presumably corresponding to primary alcohol, tertiary alcohol resulting from rearrangement, and hydrocarbon, respectively, as shown in the hydrolysates of mannosyl-1-phosphoryldecaprenol (Takayama and Goldman, 1970). However, potato phosphatase was effective for the hydrolysis of these polyprenyl pyrophosphates to the free prenols. The radiochromatogram of the hydrolysates showed two radioactivity peaks (Figure 6). The major peak with an R_f 0.42 (peak b) coincided with the spot of solanesol as a reference, supporting that this peak was associated with polyprenols. When the materials corresponding to the other radioactivity peak with an R_f 0.70 (peak a) were treated with $LiAlH_4$, the reduction product was cochromatographed with solanesol under the same conditions. Therefore, the materials corresponding to radioactivity peak a were assumed to be the aldehydes derived from the polyprenols probably by enzymatic oxidation with the potato preparations. The polyprenol fraction obtained by extraction from the section corresponding to the radioactivity peak b in Figure 6 was resolved into two components in reversed-phase chromatography on Avicel plate in solvent IV (Figure 6), showing two radioactivity peaks with nearly equal intensities. The slower moving peak (R_f 0.18) was associated with authentic solanesol. The faster moving material with an R_f 0.29 was expected to be a C_{40} polyprenol. For preparative purpose the polyprenols were purified by silica gel thin-layer chromatography followed by high-speed liquid chromatography with a porous polymer column, and two radioactive polyprenols were similarly separated (Figure 7). Two major peaks were observed as monitored by UV absorption at 215 nm at retention times of 23 and 29 min with a relative intensity of 5:3. No peak was observed in this region when the ether extracts from a control incubation with the potato phosphatase preparation without the polyprenyl pyrophosphate products were analyzed, indicating that these two peaks were fully due to the polyprenols derived from the products of the synthetase reaction and that no endogenous polyprenol was present in the phosphatase preparation. De-

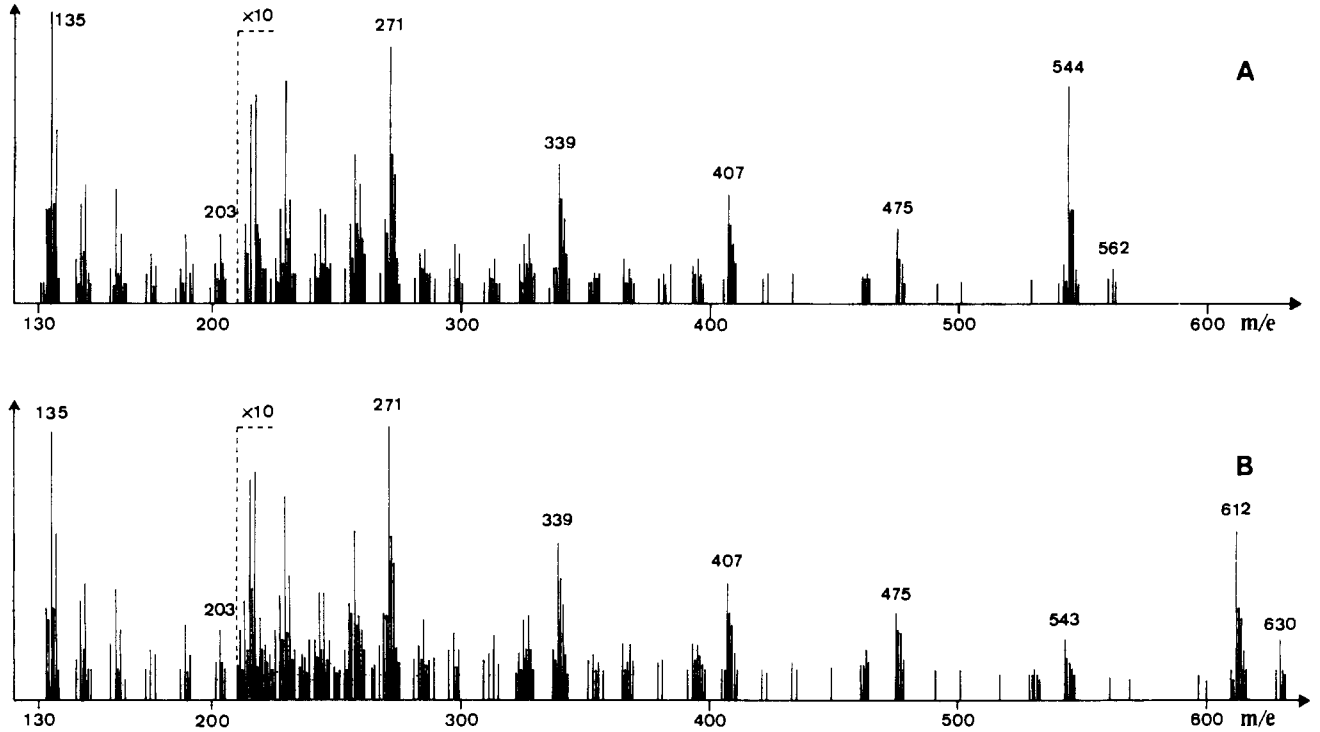

FIGURE 8: Mass spectra of the polyprenols purified by liquid chromatography. Fractions A and B obtained by high-speed liquid chromatography were concentrated and introduced on a probe at 80 °C directly into the ion source. The potential of the ionizing electron beam was 70 eV. Mass peaks above 131 are presented.

termination of the radioactivity of each fraction showed that 93% of the radioactivity in the products was associated with the fractions corresponding to these two peaks. The fractions emerging between 22 and 24 min (fraction A) and between 29 and 31 min (fraction B) were analyzed by mass spectrometry (Figure 8). Both of these fractions exhibited typical fragmentation patterns reasonable for isoprenoid alcohols of the general structure. Prominent fragments of the material in the former fraction were derived by parent peak *m/e* 562 (M) corresponding to octaprenol, $C_{40}H_{66}O$, by loss of water *m/e* 544 (M − H_2O), and subsequent elimination of terminal units in a regular manner from the ω end by cleavage at the allylic positions. The mass spectrum of the latter fraction showed peaks at *m/e* 630 (M) corresponding to nonaprenol, $C_{45}H_{74}O$, *m/e* 612 (M − H_2O), and other peaks due to subsequent elimination analogous to that of the former fraction. These results indicate that the products synthesized by the polyprenyl pyrophosphate synthetase are octaprenyl (C_{40}) and nonaprenyl (C_{45}) pyrophosphate. The possibility of formation of other prenyl pyrophosphates which might not have been hydrolyzed by the potato enzyme treatment was excluded by confirming that the total radioactivity in the polyprenol fraction obtained by the phosphatase treatment was equal to that in the hydrolysates obtained by the acid treatment of the synthetase reaction products.

The stereochemical course of the biosynthesis of these polyprenyl pyrophosphates was determined using stereospecifically labeled [2-^{3}H]isopentenyl pyrophosphate mixed with [1-^{14}C]isopentenyl pyrophosphate and geranyl pyrophosphate as a cosubstrate (Table I). The petroleum ether soluble extracts obtained by acid treatment of the polyprenyl pyrophosphates synthesized from the mixed labeled substrates were subjected to silica gel thin-layer chromatography. The radiochromatogram showed three radioactivity peaks (I, II, and III) corresponding to primary alcohol, tertiary alcohol, and hydrocarbon fractions, respectively. Any of these three fractions derived from [1-^{14}C]- and (2*R*)-[2-^{3}H]isopentenyl pyrophosphate and geranyl pyrophosphate showed no radioactivity for ^{3}H, whereas those from [1-^{14}C]- and (2*S*)-[2-^{3}H]-isopentenyl pyrophosphate and geranyl pyrophosphate showed the same ^{3}H/^{14}C ratio as that in the starting substrate. For a control experiment, *all-trans*-farnesyl pyrophosphate synthesized from the mixed-labeled isopentenyl pyrophosphate by the action of liver farnesyl pyrophosphate synthetase was also analyzed. The ^{3}H/^{14}C ratio of isopentenyl pyrophosphate was kept unchanged in the farnesyl pyrophosphate derived from [1-^{14}C]- and (2*S*)-[2-^{3}H]isopentenyl pyrophosphate and dimethylallyl pyrophosphate, whereas that derived from [1-^{14}C]- and (2*R*)-[2-^{3}H]isopentenyl pyrophosphate and dimethylallyl pyrophosphate contained no tritium. These results indicate that only the 2-*pro-R* proton of isopentenyl pyrophosphate is lost in the formation of the octaprenyl and nonaprenyl pyrophosphate and, therefore, that each isoprene unit added to geranyl pyrophosphate has a trans configuration about the newly formed double bond.

Discussion

The evidence described above shows that the present enzyme purified from extract of *M. lysodeikticus* catalyzes the formation of *all-trans*-octaprenyl (C_{40}) and *all-trans*-nonaprenyl (C_{45}) pyrophosphate. Therefore, we propose to call this enzyme solanesyl pyrophosphate synthetase as a trivial name. The role of this enzyme is presumably to supply the prenyl side chain of menaquinone-9 which is known to occur in this bacterium (Bishop et al., 1962; Fujita et al., 1966).

Allen et al. (1967) were the first to obtain a partially purified enzyme preparation that synthesizes polyprenyl pyrophosphates with the chain length of C_{35}–C_{50} from *M. lysodeikticus*. Winrow and Rudney (1969) applied the ability of this enzyme to form such polyprenyl pyrophosphates to the study of the ubiquinone biosynthesis. They showed using either the rat liver or *Rhodospirillum rubrum* preparation that the products

TABLE I: Stereospecific Incorporation of Isopentenyl Pyrophosphate into Polyprenyl Pyrophosphate.[a]

Compounds	[1-^{14}C,(2*R*)-2-^{3}H]-Isopentenyl PP[b]			[1-^{14}C,(2*S*)-2-^{3}H]-Isopentenyl PP		
	^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C	^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C
Isopentenyl PP[c]	196 000	46 200	4.17	98 700	44 400	2.23
Farnesyl PP[d]	121	8 719	0.01	16 637	7 477	2.22
Polyprenyl PP						
I	5	4 433	0.00	8 760	3 977	2.20
II	8	7 563	0.00	14 773	6 700	2.20
III	0	1 869	0.00	4 021	1 910	2.10

[a] The reaction mixture contained, in a final volume of 2.0 mL, 200 μmol of Tris-HCl buffer (pH 7.4), 5 μmol of $MgCl_2$, 40 nmol of geranyl pyrophosphate, 18 nmol of [1-^{14}C,(2*R*)-2-^{3}H]isopentenyl pyrophosphate or 17 nmol of [1-^{14}C,(2*S*)-2-^{3}H]isopentenyl pyrophosphate, and DEAE-Sephadex purified enzyme (0.48 mg). After 2 h of incubation, the mixture was treated in the same manner as described in the standard assay, except that petroleum ether was used for extraction instead of hexane. The extracts were subjected to silica gel thin-layer chromatography in solvent II. Thin-layer chromatography separated the hydrolysates into three components (I, II, and III, R_f 0.44, 0.57, and 0.71, respectively), which were extracted with ether and assayed for radioactivity in toluene scintillation fluid. [b] PP stands for pyrophosphate. [c] The radioactivity of mixed-labeled isopentenyl pyrophosphate was determined as follows: The mixed-labeled isopentenyl pyrophosphate was chromatographed on a silica plate in solvent I. The radioactive isopentenyl pyrophosphate was extracted with water from the section corresponding to the radioactivity peak (R_f 0.14) and was hydrolyzed with alkaline phosphatase. The resulting isopentenyl alcohol was extracted with ether and counted for ^{3}H and ^{14}C radioactivity in toluene scintillation fluid. [d] For the reference experiment, the condensation of the mixed-labeled isopentenyl pyrophosphate with dimethylallyl pyrophosphate was performed by farnesyl pyrophosphate synthetase purified from pig liver. Radioactive *all-trans*-farnesol obtained by the hydrolysis with alkaline phosphatase was purified on a silica plate in solvent III and counted for radioactivity in toluene scintillation fluid.

obtained by incubation of isopentenyl pyrophosphate with an extract of *M. lysodeikticus* served as substrates for enzymatic prenylation of *p*-hydroxybenzoate to give 4-carboxy-2-polyprenylphenols with the side chain ranging from C_{35} to C_{50} (Momose and Rudney, 1972). Similar results were also reported in the study of the prenylation of the ubiquinone precursor using broad bean and yeast preparations, indicating that the *M. lysodeikticus* extract provided polyprenyl pyrophosphates of C_{30}–C_{50} (Thomas and Threlfall, 1973). These results suggest that the products derived from isopentenyl pyrophosphate by the action of the *M. lysodeikticus* enzyme contain *all-trans*-polyprenyl pyrophosphates.

The enzyme reported here catalyzes the formation of all-trans C_{40} and C_{45} prenyl pyrophosphate. Although several attempts were made to change the reaction conditions so that a different distribution of the products might be observed, neither a shorter nor longer product than these two was formed in an appreciable amount. It is not clear whether the formation of C_{35} and C_{50} prenyl pyrophosphate is attributed to enzyme other than the present solanesyl pyrophosphate synthetase, but it may be probable that a single enzyme primarily responsible for the synthesis of solanesyl pyrophosphate forms a minor amount of C_{35} and C_{50} prenyl pyrophosphate under certain conditions because of the tolerant specificity of the enzyme. Pure farnesyl pyrophosphate synthetase is known to catalyze the formation of not only geranyl and farnesyl pyrophosphate but also geranylgeranyl pyrophosphate under strengthened conditions (Reed and Rilling, 1975).

A previous work from this laboratory has shown that an extract of fresh cultures of *M. lysodeikticus* catalyzes the synthesis of undecaprenyl phosphate (C_{55}) which presumably has cis,trans mixed stereochemistry (Kurokawa et al., 1971). It is reasonable to assume that the formation of the undecaprenyl phosphate is attributed to another enzyme.

Allen et al. (1976) have succeeded in partial purification from *Lactobacillus plantarum* of undecaprenyl pyrophosphate synthetase catalyzing the cis addition of isopentenyl pyrophosphate giving rise to the sugar-carrier lipid, the undecaprenyl pyrophosphate of cis,trans mixed stereochemistry. The undecaprenyl pyrophosphate synthetase is known to show a fivefold preference for *all-trans*-farnesyl pyrophosphate over geranyl pyrophosphate, whereas dimethylallyl pyrophosphate is not accepted as substrate. On the other hand, the solanesyl pyrophosphate synthetase described in this paper is much more active with geranyl pyrophosphate rather than with *all-trans*-farnesyl and *all-trans*-geranylgeranyl pyrophosphate and inactive with dimethylallyl pyrophosphate or *cis,-trans,trans*-geranylgeranyl pyrophosphate. It is very interesting that dimethylallyl pyrophosphate, which is the primer in the chain elongation catalyzed by general prenyltransferases, cannot act as a starting substrate for either of these two polyprenyl pyrophosphate synthetases. This implies that a mechanism of supply of allylic pyrophosphate other than dimethylallyl pyrophosphate must be involved in the biosynthesis of polyprenyl pyrophosphates. Geranylgeranyl pyrophosphate synthetase which is known to be present in *M. lysodeikticus* and catalyzes the synthesis of *all-trans*-geranylgeranyl pyrophosphate from isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Kandutsch et al., 1964) may be responsible for this mechanism. However, our preliminary study has revealed the presence in this bacterium of a new prenyltransferase providing geranyl pyrophosphate. Consequently, it seems more conceivable from the high effectiveness of geranyl pyrophosphate that this enzyme is responsible for the supply of the starting substrate for the solanesyl pyrophosphate synthetase. The study of the new prenyltransferase will be reported elsewhere.

The difficulty in the structural study of polyprenyl pyrophosphates had been largely due to their resistant property against phosphatase. Yeast and liver homogenates cleave polyprenyl monophosphates but they are not effective or poorly active to cleave the polyprenyl pyrophosphates (Kurokawa et al., 1971; Allen et al., 1976). The fact that potato leaves contain a considerable amount of solanesol (Toyoda et al., 1969) led us to examine a potato preparation for a better phosphatase, and we found that a low-speed supernatant from young shoots acted effectively in the presence of a detergent on these polyprenyl pyrophosphates to give the corresponding prenols. This finding will be widely useful for the structural study of polyprenyl pyrophosphates.

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Physical Evidence for an Apolar Binding Site Near the Catalytic Center of Human α-Thrombin†

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ABSTRACT: Proflavin dye displacement studies, electron spin resonance active spin label studies and Tos-Arg-OMe esterase rate measurements on human α-thrombin have shown the presence of an apolar binding site for indole which may reside quite near the active center. Indole displaced proflavin from its binding locus at the thrombin active site with a dissociation constant K_{In} = 10.6 ± 1.3 mM (pH 6.5, 0.05 M sodium phosphate, 0.75 M NaCl, 25 °C) as measured by the decrease in the 468-nm difference spectral maximum for the human α-thrombin–proflavin complex. Only 3 isomorphous fluorosulfonylphenyl spin-labeled thrombins of a broad series were effected by indole binding whereas all 14 in the series were altered in their rotational mobility upon binding basic ligands such as benzamidine or *p*-chlorobenzylamine. This confirmed that indole binding was localized to a unique site in the active site region. Tos-Arg-OMe esterase activity of human α-thrombin was activated by indole while clotting activity was unaltered. The Tos-Arg-OMe activation was observed if the substrate concentration (1 mM) remained below those concentrations where (bovine) thrombin normally displayed substrate activation (e.g., 5 mM Tos-Arg-OMe). At 5 mM Tos-Arg-OMe indole did not activate the hydrolytic rate. These rate effects were examined in both no salt and in 0.3 M NaCl. The esterase activity was fourfold greater in the *absence* of NaCl. The apparent activation equilibrium dissociation constant for indole at pH 8.1 in *no* salt, 25 °C, was K_{In} = 4.3 ± 1.8 mM. The same constant in 0.3 M NaCl was not accurately measurable due to solubility problems but was approximately 10–50 mM. A model is proposed which places the indole binding site no farther than a proflavin molecular length from the basic substrate binding pocket.

Thrombin (EC 3.4.21.5) is a unique seryl protease in its (apparently) specific interactions at the physiological level with, e.g., its primary peptidyl substrate, fibrinogen, with platelets, and with several other coagulation proteins in serum (factors II, V, VIII, XIII, etc.). It is generated by proteolytic cleavages of its circulating zymogen (prothrombin or coagulation factor II) through a unique complex of several blood and tissue factors. Thus this enzyme is a serine protease of restricted "trypsin like" specificity yet functions *specifically* in several hemostatic events. Despite the importance of human thrombin, comparatively little is known about this latter species relative to the bovine enzyme, upon which most of the past structure–function work on thrombin has been focused. With the recent availability of isolation procedures for human thrombin of high purity and activity, this protein is now amenable to physicochemical investigations at the molecular level (Fenton et al., 1977a,b).

The importance of apolar or hydrophobic binding regions

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