FORMATION OF POLYPRENYL PHOSPHATES BY A CELL-FREE ENZYME OF MICROCOCCUS LYSODEIKTICUS

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SUMMARY

A cell-free enzyme of Micrococcus lysodeikticus catalyzed the formation of polyprenyl phosphates ranging in carbon chain from C-20 to C-55 with a predominance of C-40. Homogenates of rabbit liver (or kidney) and yeast catalyzed effectively the hydrolysis of these long chain prenyl phosphates but did not act on the pyrophosphate esters.

Allen et al., reported a partial purification of long chain prenyl pyrophosphate synthetase from Micrococcus lysodeikticus which catalyzes the conversion of isopentenyl pyrophosphate plus allylic pyrophosphate such as farnesyl pyrophosphate into a mixture of polyprenyl pyrophosphates ranging in carbon chain from C-35 (heptaprenyl) to C-50 (decaprenyl), though detailed information on the component and structure of the products was not available (1). In the meanwhile, undecaprenyl phosphate (C-55) has been found to be implicated in the biosynthesis of peptidoglycan and mannan in the same bacteria (2, 3). However, the significance of this long chain prenyl pyrophosphate synthetase in relation to the undecaprenyl phosphate is not known.

This paper reports the finding that a crude enzyme of M. lysodeikticus catalyzes the conversion of isopentenyl pyrophosphate plus farnesyl pyrophosphate into undecaprenyl phosphate as well as its lower isoprenologs and that these long chain prenyl phosphates are dephosphorylated effectively by the action of homogenate of rabbit liver (or kidney) or yeast to give the corresponding prenols.

MATERIALS AND METHODS

M. lysodeikticus (IAM 1056) was grown in the medium of Lennarz et al., (4) at 27° for 4 days. Cells were collected and washed with 0.05M Tris-HCl buffer, pH 7.4. The cells (45 g, wet wt) were suspended in 400 ml of the same buffer containing lysozyme (100 mg), and the mixture was stirred at room temperature for 30 min. After the treatment with DNase (40 mg) for 10 min, the mixture was centrifuged at 77,000 x g for 60 min. The supernatant was fractionated with $(NH_4)_2SO_4$, and the fraction precipitating between 30 and 50% saturation was dissolved in 0.05M Tris-HCl buffer, pH 7.4. The resulting solution was dialyzed against the same buffer. The dialyzed solution and the 77,000 x g supernatant were used as the crude enzyme, and the result was almost the same regardless of the enzyme used. The long chain prenyl pyrophosphate synthetase was purified from this crude enzyme fraction according to the method of Allen et al. (1). The homogenate of rabbit liver (or kidney) was prepared as follows : The tissue (45 g, wet wt) was minced with scissors in 0.05M Tris-HCl buffer, pH 7.4 and washed several times with the same buffer. The mince was ground in a mortar with sea sand in 60 ml of the buffer to a homogenate, which was filtered through gauze and centrifuged at 500 x g for 20 min. The resulting supernatant was used for the study of the dephosphorylation. Baker's yeast (15 g) was ground with sea sand in 5 ml of the same buffer for 10 min to a homogenate, which was then centrifuged at 500 x g for 10 min. The supernatant was used as the enzyme for the dephosphorylation. The preparation of the enzymes described above was carried out at 4° unless otherwise stated.

Farnesyl pyrophosphate was prepared by the phosphorylation of trans,trans-farnesol by the method of Kandutsch et al. (5).

^{14}C-Isopentenyl pyrophosphate (1.2Ci/mole) was the same preparation as in the previous study (6).

The incubation conditions are given under the Figures.

RESULTS AND DISCUSSION

Ambiguity is unavoidable in the published results (1) of the products by the long chain prenyl pyrophosphate synthetase for the reason that the products were not cleaved by phosphatase to prenols suitable for the analysis and that a complex mixture of dephosphor-

ylated materials obtained by the acid treatment was subjected to their analytical experiment. In order to study the component and structure of the products it is desirable to study a method of suitable dephosphorylation of the products. It is also reported that the products by the synthetase bind strongly to the enzyme protein (1). Therefore, we tried first to separate the products from the enzyme, and it was found that the long chain prenyl pyrophosphates could be isolated from the enzyme by $CHCl_3$-CH_3OH (2 : 1) or n-butanol extraction of a mixture obtained according to the procedure of Allen et al. (1). However, thus obtained enzyme free products were also resistant to alkaline phosphatase, and they emerged in the V_0 region in the Sephadex G-25 filtration. These facts indicate that the particular behavior of the products (1) is not necessarily due to the binding but to a property of long chain prenyl pyrophosphate. Reductive dephosphorylation of the products with $LiAlH_4$ gave a petroleum soluble material , but it was not suitable for further analysis because the material was a mixture of alcohols and hydrocarbons with a predominance of the latter.

Then an enzymic dephosphorylation was attempted. It has been reported that presqualene pyrophosphate is hydrolyzed to a C-30 alcohol by rat liver or yeast microsomes (7). We examined the action of rabbit liver (or kidney) and yeast homogenates on the long chain prenyl pyrophosphates synthesized by the purified synthetase of M. lysodeikticus, but no appreciable hydrolysis was observed. However, an effective hydrolysis was observed when the product by the crude enzyme was used. The product by the crude enzyme behaved similar to that by the purified synthetase in that it was extractable with $CHCl_3$-CH_3OH or n-butanol and not hydrolyzed by alkaline phosphatase and that it emerged in the V_0 region in Sephadex G-25 filtration. However, comparison of these two pro-

ducts on TLC clearly indicated that the product by the crude enzyme was not pyrophosphate but monophosphate ester. The products by the purified and the crude enzyme had Rf value of 0.36-0.40 and 0.65-0.80, respectively on silica gel with isopropanol-ammonia-water (6 : 3 : 1) in which the Rf's for geranylgeranyl pyrophosphate and its monophosphate were 0.20 and 0.56, respectively. In the cycle of the peptidoglycan biosynthesis there is known to be involved a step for undecaprenyl pyrophosphate to be cleaved to the monophosphate ester (8). It is probable that the similar enzyme activity is included in the crude enzyme preparation in this experiment. Thus the liver (or kidney) and yeast homogenates were found to catalyze the hydrolysis of the long chain prenyl phosphate.

Fig. 1 shows a thin layer radiochromatogram of the materials extracted with ether from the reaction mixture treated with the rabbit liver homogenate. The material corresponding to peak A has the same Rf value as that for solanesol in any of the following solvent systems; benzene-petroleum ether(22:78), benzene-ethyl acetate(9:1) and petroleum ether-acetone(9:1), and the Rf is in good accord with that for a minor product obtained by $LiAlH_4$ treatment of the pyrophosphate esters synthesized by the purified enzyme. These facts suggest that the peak A is associated with polyprenol similar to solanesol. Then the radioactive material in the region under the peak A was extracted with ether and rechromatographed on a reversed phase TLC which clearly separated a mixture of betulaprenols* to each component. As obvious in Fig. 2, major radioactivity is associated with C-40 and C-45 prenol, and the radioactivity corresponding to C-35 and C-30 is minor. When the same sample was subjected

* Betulaprenols (C-30 to C-45) were isolated from Betula schmidtii ("Onoore" in Japanese) essentially according to the procedure reported by Lindgren (9). The details will be reported elswhere.
** Purification of geranylgeranyl pyrophosphate synthetase has been already reported (5).

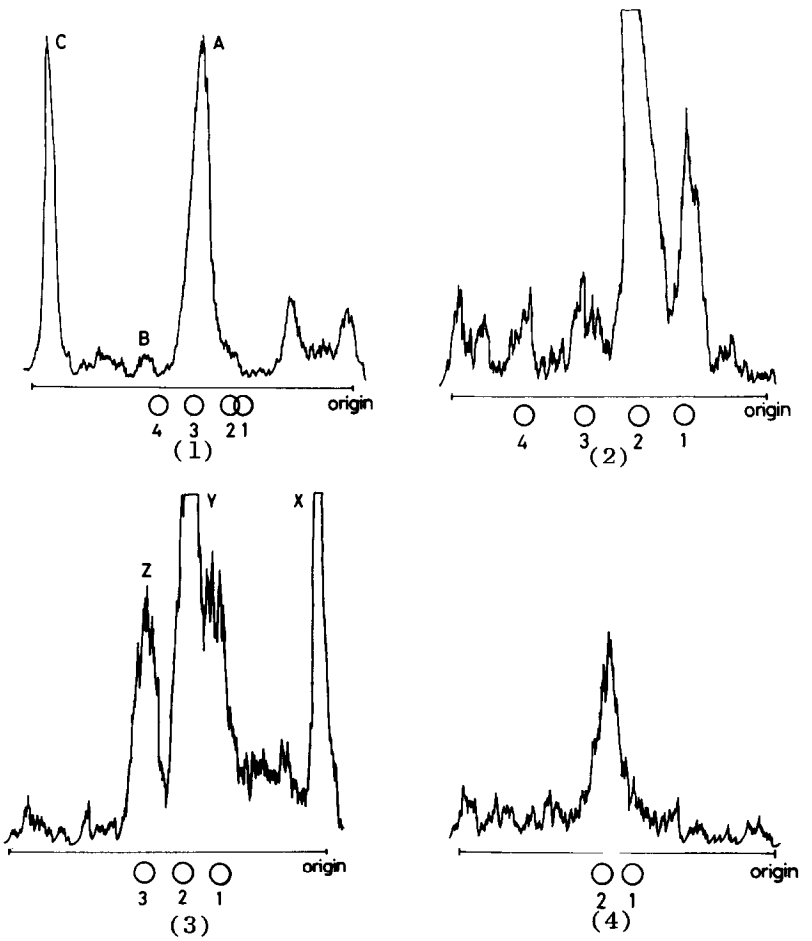

Fig. 1. TLC of the products by the treatment with liver homogenate. The incubation mixture contained, in a final volume of 0.8 ml, Tris-HCl, pH 7.4, 100 μmoles; $MgCl_2$, 5 μmoles; farnesyl pyrophosphate, 0.0025 μmole; and radioacitve isopentenyl pyrophosphate, 0.05 μmole and the 77,000 x g supernatant of M. lysodeikticus, 0.4 ml. After the incubation at 37° for 5 hr, the rabbit liver homogenate, 0.3 ml was added, and the mixture was incubated at 37° for 5 hr. Then the mixture was extracted with ether, and the extracts were chromatographed on a silica gel plate with benzene-ethyl acetate(9:1). Spots of references: 1, farnesol; 2, geranylgeraniol; 3, solanesol; 4, C-55 and C-60 prenols

Fig. 2. Reversed phase TLC of the materials extracted from the section corresponding to the peak A in Fig. 1. A plate coated with Avicel SF (microcrystalline cellulose) impregnated with liquid paraffin was used in a system of acetone-water(17:3) saturated with paraffin. Spots of reference prenols: 1, C-45; 2, C-40; 3, C-35; 4, C-30 prenol.

Fig. 3. TLC of the products by the treatment with yeast homogenate. The condition was the same as that described under Fig. 1, except that yeast homogenate was used in place of liver homogenate. Spots of references: 1, geranylgeraniol; 2, solanesol; 3, C-55 and C-60 prenols.

Fig. 4. Reversed phase TLC of the material extracted from the section corresponding to the peak Z in Fig. 3. Solvent: acetone-water(19:1) saturated with paraffin. Spots of references: 1, C-60 prenol; 2, C-55 prenol.

to another TLC in a system of acetone-water(9:3) which is suitable for the separation of C-20 to C-30 prenols, the coincidence of radioactivity with spots of C-20 and C-30 prenol was confirmed;** and besides the presence of C-25 prenol was also suggested though the peak was small. The peak B (Fig. 1) corresponding to the spot of a mixture of C-55 and C-60 prenols isolated from silkworm feces (10) was observed more distinctly in another experiment with yeast homogenate which is described later. The material corresponding to the peak C remained at the origin when developed with n-hexane-benzene(9:1), indicating that this was not for hydrocarbon. The radioactive material was extracted with ether from the section corresponding to the peak C and treated with $LiAlH_4$ to give a material which was found to behave quite similar to the material corresponding to the peak A, suggesting that the peak C is attributed to the aldehydes derived enzymically from the prenols. The remaining peaks near to the origin (Fig. 1) are not identified. A control incubation of ^{14}C-isopentenyl pyrophosphate and farnesyl pyrophosphate with the liver homogenate indicates that these peaks are probably due to materials formed by the action of the liver homogenate independently of the enzyme of M. lysodeikticus.

Yeast homogenate was found also to catalyze the hydrolysis of the long chain prenyl phosphates. Fig. 3 is a radiochromatogram of the products of the hydrolysis. The peak Y and Z correspond essentially to the peak A and B in Fig. 1, respectively. Rechromatography of the material under the peak Z on a reversed phase TLC revealed that the radioactive material was a C-55 prenol. The peak X is probably due to a material formed by the action of the yeast homogenate independently of the enzyme of M. lysodeikticus. The amount of the formation of the C-55 prenol relative to that of the other prenols appeared to vary with different enzyme preparations. The cis,trans configuration of each of these polyprenols is now under investigation.

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