Essential protein factors for polyprenyl pyrophosphate synthetases

Separation of heptaprenyl pyrophosphate synthetase into two components

H. Fujii[+], T. Koyama and K. Ogura*

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

Received 14 July 1983

Heptaprenyl pyrophosphate synthetase from *Bacillus subtilis* was dissociated into two essential components, none of which had catalytic activity alone. The enzyme activity was restored when the two components were combined with each other. Both fractions, designated components I and II in the order of their elution from DEAE–Sephadex, appeared to be proteins of M_r 30000. Component I was much more stable than component II which was easily destroyed by relatively mild heat treatment. Neither was interchangeable with any of the essential components of hexaprenyl pyrophosphate synthetase of *Micrococcus luteus* B-P 26.

Heptaprenyl pyrophosphate synthetase Bacillus subtilis *Polyprenyl pyrophosphate Prenyltransferase* *Polyprenol*

1. INTRODUCTION

In contrast to the fatty acid biosynthesis in which acyl carrier protein plays a central role [1], there is no evidence to show that such a coenzymatic protein is involved in the biosynthesis of polyisoprenoids. However, we recently separated from *Micrococcus luteus* B-P 26 two dissimilar protein fractions, components A and B, each of which had no catalytic activity at all, but catalyzed the synthesis of all-*trans*-hexaprenyl pyrophosphate when combined with each other [2]. We also showed that component A was smaller in size and more heat-stable than component B [2]. These findings stimulated us to investigate whether the requirement of such two protein components is a general matter for the synthesis of polyprenyl pyrophosphates, and, if so, whether the two components correspond to an enzyme and its essential factor of coenzymatic nature or two easily separable subunits of an enzyme system.

Authors in [3] found and partially purified from *Bacillus subtilis* heptaprenyl pyrophosphate synthetase catalyzing the synthesis of all-*trans*-heptaprenyl pyrophosphate. They also showed that the enzyme lost its activity when chromatographed on DEAE–Sephadex [3]. This fact led us to a further study on the heptaprenyl pyrophosphate synthetase with special attention to such essential components as mentioned above. This paper concerns the separation and properties of the essential components of the heptaprenyl pyrophosphate synthetase.

2. MATERIALS AND METHODS

[1-^{14}C]Isopentenyl pyrophosphate and non-

[+] Present address: Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980, Japan

* To whom correspondence should be addressed

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labelled allylic pyrophosphates were the same preparations as in [2].

2.1. *Cell culture*

Cells of *B. subtilis* 111 were grown at 37°C on a nutrient broth (Oxoid) and 1 liter of distilled water; 3 l shaking flasks, each containing 500 ml of medium, were inoculated with 15 ml of a preincubated suspension from a 12-h shaking culture. After 20 h of growth with shaking, cells were harvested by centrifugation and washed twice with 50 mM Tris–HCl buffer (pH 7.4), and used for the enzyme preparation.

2.2. *Separation of component I and component II*

Cells (wet wt, 10 g) were suspended in 70 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 50 mM KCl (buffer A). Lysozyme (130 mg) was added to the cell suspension, and the mixture was allowed to stand for 1 h at room temperature with stirring. The lysate was then treated with DNase (10 mg) for 10 min at room temperature and centrifuged at 30900 × 9 for 90 min. Subsequent steps were carried out at 4°C. The supernatant fraction of centrifugation at 30900 × *g* was chromatographed on a DEAE–Sephadex A-50 column (2.8 × 39 cm) equilibrated with buffer A. Elution was performed with a linear gradient of 50–850 mM KCl. The fractions eluted from the DEAE–Sephadex A-50 column were used for the experiment to search for essential components of heptaprenyl pyrophosphate synthetase. Protein concentration was estimated by measuring absorbance at 280 nm.

2.3. *Assay of heptaprenyl pyrophosphate synthetase activity*

The incubation mixture contained, in a final volume of 1.0 ml, 100 μmol *N*-Tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)–NaOH buffer (pH 7.4), 5 μmol $MgCl_2$, 5 μmol potassium fluoride, 25 nmol all-*trans*-farnesyl pyrophosphate, 25 nmol [1-^{14}C]isopentenyl pyrophosphate (spec. act. 1.0 Ci/mol) and an enzyme fraction. The enzyme activity was measured and expressed as the radioactivity incorporated into the product as in [3].

2.4. *Product analysis*

The polyprenyl pyrophosphate formed by the enzymatic reaction was hydrolyzed by the acid phosphatase method [4], and the polyprenol liberated was identified as in [5].

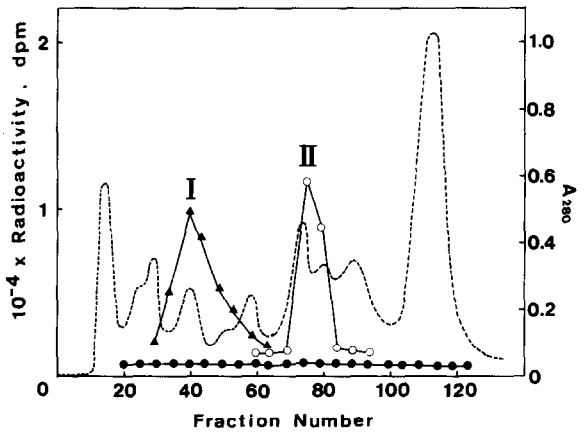

Fig.1. Separation of two essential components of heptaprenyl pyrophosphate synthetase by DEAE–Sephadex chromatography. The heptaprenyl pyrophosphate synthetase activity was measured with a supplement of Fr-a (○), Fr-b (▲) or without the supplement (●). (– – –) Absorbance at 280 nm.

3. RESULTS AND DISCUSSION

As in [3], prenyltransferase activities including heptaprenyl pyrophosphate synthetase were detected in a crude extract of *B. subtilis* when assayed with [1-^{14}C]isopentenyl pyrophosphate and either dimethylallyl-, geranyl-, all-*trans*-farnesyl- or all-*trans*-geranyl-geranyl pyrophosphate. However, when the extract was chromatographed on a DEAE–Sephadex column, the heptaprenyl pyrophosphate synthetase was not detected in any fraction as shown by the closed circles in fig.1, whereas farnesyl pyrophosphate synthetase was recovered in fractions 65–75 (not shown). As the loss of the heptaprenyl pyrophosphate synthetase activity seemed too marked to be attributed to denaturation of the enzyme, we examined whether the synthetase activity could be restored by recombining the chromatographically separated fractions. First, the fractions eluted from the DEAE–Sephadex A-50 column were combined to make 3 major fractions as follows: Fr-a, fractions 20–61; Fr-b, fractions 62–78; Fr-c, fractions 79–120. Each fraction was concentrated by ultrafiltrations through a PM-10 membrane with an ex-

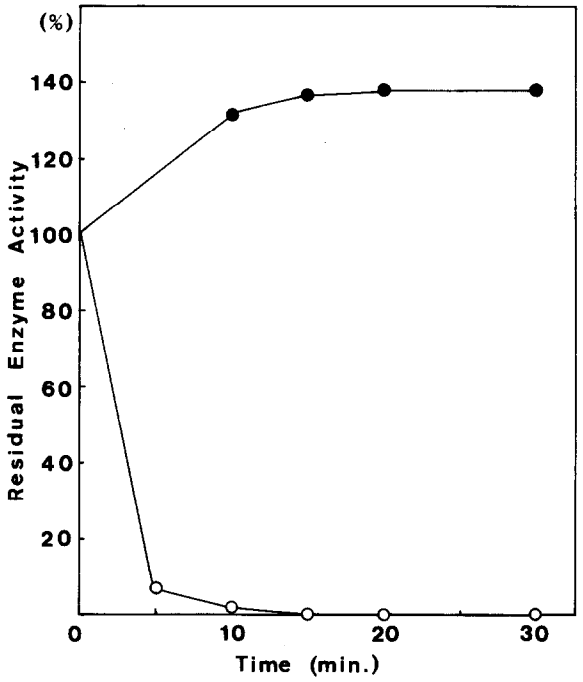

Fig.2. Heat stability of components I and II. The Sephadex G-100 purified component I (●) and component II (○) were incubated at 50°C for the indicated period, and then assayed for the restorative activity for heptaprenyl pyrophosphate synthetase with a supplement of untreated component II and untreated component I, respectively.

clusion limit of $M_r = 10000$ under a nitrogen atmosphere. Neither of these 3 fractions alone, nor a combination of Fr-b and Fr-c was significantly active as enzyme. However, either Fr-b or Fr-c exhibited heptaprenyl pyrophosphate synthetase activity when combined with Fr-a. In order to locate in more detail the chromatographic elution of the substance responsible for this restorative activity, the eluates were assayed with [1-^{14}C]isopentenyl pyrophosphate and all-*trans*-farnesyl pyrophosphate in the presence of Fr-a or Fr-b. As a result, a remarkable peak for polyprenyl pyrophosphate synthetase activity appeared in a region of fractions 70–85 when assayed in the presence of Fr-a (fig.1). When the eluates of the same chromatography were assayed in the presence of Fr-b, in turn, another peak for the heptaprenyl pyrophosphate synthetase activity appeared in a region of fractions 30–60. The product of the reconstitutive incubation was all-*trans*-heptaprenyl pyrophosphate. Thus, it was demonstrated that the complete loss of heptaprenyl pyrophosphate synthetase activity by DEAE–Sephadex chromatography was due to the resolution of the enzyme into two essential fractions which themselves were catalytically inactive unless combined. The essential components in fractions 30–60 and fractions 70–85 in fig.1 are designated as component I and component II, respectively. These components were further purified 2–3-fold by Sephadex G-100 filtration (not shown). From the gel filtration, both their M_r-values were estimated to be 30000. In spite of the similarity of their size, they were very different in stability. As shown in fig.2, component I was more stable against heat treatment than component II. The dependency of heptaprenyl pyrophosphate synthetase activity on the concentration of component I is given in fig.3.

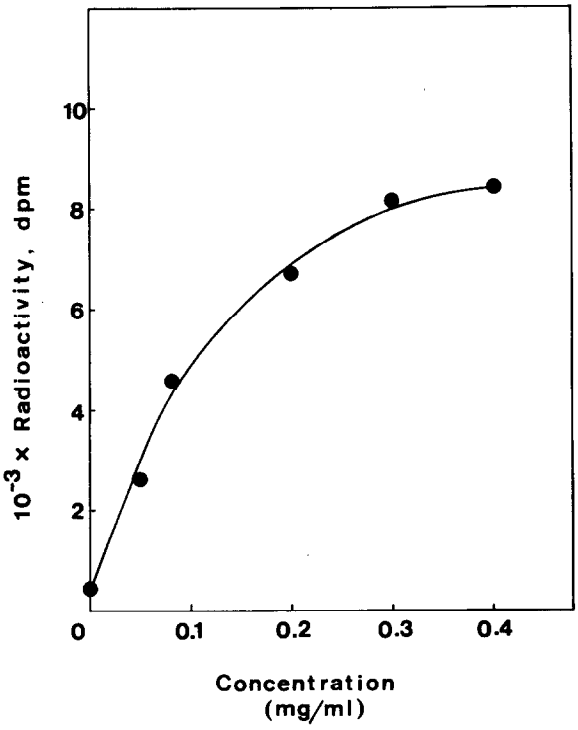

Fig.3. Effect of the concentration of component I on the heptaprenyl pyrophosphate synthetase activity. Enzyme activity was measured as described in section 2. Component II (0.84 mg) was incubated with [1-^{14}C]isopentenyl pyrophosphate and all-*trans*-farnesyl pyrophosphate in the presence of component I at various concentrations.

Table 1

Assay for prenyltransferase activity of various combinations of the components of hexaprenyl pyrophosphate and heptaprenyl pyrophosphate synthetase

Combination	Enzyme activity (dpm)
Component I + component II	2525
Component I + component B	132
Component A + component II	409
Component I + component A	258
Component II + component B	158
Component A + component B	3172
Component A alone	283
Component B alone	216
Component I alone	140
Component II alone	150

Components A and B were obtained as in [3]. The amounts of protein used for this assay were: 0.9 mg (component I); 0.3 mg (component II); 0.3 mg (component A); 0.16 mg (component B)

Both of the two components were shown to be proteins by an experiment of trypsin treatment carried out similarly to that in [2].

Our next interest was to know whether each of the two components is specific for its own counterpart or interchangeable with component A or B of the hexaprenyl pyrophosphate synthetase of *M. luteus* B-P 26 [2]. We assayed all the 4 possible hybrid mixtures, and found that none of these showed any appreciable synthetase activity (table 1).

These results eliminate the involvement of a common factor to polyprenyl pyrophosphate synthetases of different specificity in the chain length of the product.

Hexaprenyl PP Synthetase:

$$C_{15}\ PP + 3\times \text{Isopentenyl PP} \xrightarrow{\text{component A + component B}} C_{30}\ PP$$

Heptaprenyl PP Synthetase:

$$C_{15}\ PP + 4\times \text{Isopentenyl PP} \xrightarrow{\text{component I + component II}} C_{35}\ PP$$

It would also be interesting to compare the essential components of prenyltransferases catalyzing the same reaction, but from different sources.

ACKNOWLEDGEMENTS

We are grateful to Dr Kazuo Izaki, Department of Agricultural Chemistry of this University for his generous gift of a cell culture of *Bacillus subtilis* Marburg strain 111. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and a grant from Sankyo.

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