Partial Purification and Characterization of 4-Hydroxybenzoate-polyprenyltransferase in Ubiquinone Biosynthesis of *Pseudomonas putida*

Kazuyoshi KAWAHARA,[†] Naohisa KOIZUMI,^{††} Haruhiko KAWAJI,^{†††}
Kunio OISHI,^{††††} Kô AIDA^{†††††} and Kinya UCHIDA*

*Institute of Applied Microbiology, The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan*

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4-Hydroxybenzoate-polyprenyltransferase, an enzyme in ubiquinone biosynthesis, from *Pseudomonas putida* was partially purified by ion-exchange and gel filtration column chromatography. The enzyme required phospholipid as an essential factor for activity. Hexaprenyl pyrophosphate(-PP) and pentaprenyl-PP as well as nonaprenyl-PP were used as polyprenyl donors, but tetraprenyl- and farnesyl-PPs were scarcely transferred to 4-hydroxybenzoic acid. No inhibition was observed by the end-product, ubiquinone-9 of *P. putida*. Long chain acyl-CoA, free fatty acids, or isopentenyl-PP strongly inhibited the enzyme activity. A possible regulatory role of the enzyme in bacterial ubiquinone biosynthesis is discussed.

In the pathway of ubiquinone biosynthesis, 4-hydroxybenzoate-polyprenyltransferase (4HB-PP transferase) is presumed to be a key enzyme which may regulate the flow of the whole synthetic pathway, and may decide the proper chain length of the ubiquinone.^{1,2)} However, because of the difficulties of purification and handling, few biochemical studies have been done on this enzyme until now. In our preceding paper,³⁾ this enzyme was solubilized from the membrane fraction of *Pseudomonas putida*, and some enzymatic characteristics were discovered. Certain phospholipids, especially phosphatidylethanolamine could activate the solubilized enzyme, suggesting that this enzyme forms a complex with phospholipids in its active form. Further

purification is also necessary to investigate the characteristics of this enzyme as a regulator of the ubiquinone biosynthesis.

In this study, 4HB-PP transferase was partially purified, and the substrate specificity and effects of lipid metabolites were clarified.

Materials and Methods

Bacterial strains and growth medium. *Pseudomonas putida* IAM1219 was used as a source of the enzyme. The bacteria was cultivated with the conditions and the medium described in our preceding paper.³⁾

Assay system of the enzyme activity. The activity of 4HB-PP transferase was assayed in 1 ml of a reaction mixture containing 10 μ M (0.5 μ Ci) 4-hydroxy[7-¹⁴C]benzoic acid, 30 μ M nonaprenyl pyrophosphate (solanesyl-PP),

* Address correspondence to this author.

[†] Present address: The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan.

^{††} Present address: Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222, Japan.

^{†††} Present address: Research Laboratories of Nippon Chemipher, 1-22 Hikokawato, Misato, Saitama 341, Japan.

^{††††} Present address: College of Agriculture and Veterinary Medicine, Nihon University, 3-34-1 Shimouma, Setagaya-ku, Tokyo 154, Japan.

^{†††††} Present address: Faculty of Domestic Science, Otsuma Women's University, 12 Sanbancho, Chiyoda-ku, Tokyo 102, Japan.

1 mM MgSO_4 , 1 mM dithiothreitol, 1.4 mM phospholipid, and the enzyme dissolved in 50 mM imidazole buffer (pH 7.0) containing 0.3% Triton X-100. The reaction product was extracted in the chloroform layer and a sample was counted by a liquid scintillation counter as described in our preceding paper.³⁾

Purification procedure for the enzyme. The solubilized enzyme (crude enzyme, 60 ml) was prepared from 30 g (wet weight) of the bacterial cells by the method in our preceding paper. The crude enzyme (57 ml) was immediately put on a DEAE-Sepharose CL-6B column (2×10 cm) and eluted with 120 ml of imidazole buffer (50 mM, pH 7.0, 0.3% Triton X-100). Adsorbed protein was eluted with 200 ml of imidazole buffer with a gradient of NaCl from 0 to 0.3 M. Fractions of high enzyme activity were combined, and polyethylene glycol 6000 (PEG) was added to 25% (w/v) in final concentration. The solution was stirred at 0°C for 30 min and the precipitate was collected by centrifuging at $100,000 \times g$. The precipitate was dissolved in 3 ml of imidazole buffer containing 15% (w/v) sucrose, and kept at -80°C . Two ml of this preparation was chromatographed on a gel filtration column with Sephacryl S-400 (2×40 cm), and eluted in 4-ml fractions. Fractions with high enzyme activity were combined (12 ml), and used for the experiments as the purified enzyme preparation.

Measurement of protein. Protein was measured by the method of Bradford⁴⁾ or the modification of the Lowry method by Sugawara.⁵⁾

Preparation of phospholipid. Cellular lipids were extracted from the lyophilized cells of *P. putida* IAM 1219 with chloroform-methanol (2:1, v/v). The extract, after being concentrated, was precipitated with 9 volumes of chilled acetone. The precipitate dissolved in chloroform-methanol (2:1, v/v) was estimated for the phospholipids by measurement of phosphorus.⁶⁾ This preparation was used for the enzyme assay, after solvent was removed by an N_2 stream and dispersed in a buffer by sonication.

Preparation of quinones. Ubiquinone with a 10-unit isoprenoid side chain (UQ_{10}), with a 9-unit side chain

(UQ_9), and with an 8-unit side chain (UQ_8) were extracted from lyophilized cells of *Pseudomonas diminuta* IAM 1513, *P. putida* IAM 1219 and *Shewanella putrefaciens* IAM 1510, respectively, by the method in our previous paper.⁷⁾ Menaquinone with a 9-unit side chain (MK_9) was a kind gift of Eizai Co. (Tokyo, Japan). They were purified by high performance liquid chromatography (HPLC) with an ODS column if necessary.

Chemicals. Nonaprenol (solanesol), hexaprenol (farnesylfarnesol), pentaprenol (geranylgeraniol), tetraprenol (geranylgeraniol), farnesol, and isopentenol were kind gifts from I. Kawada of Takasago Perfumery Co. (Tokyo, Japan). The purity was confirmed by gas chromatographic analysis. Phytol was obtained from Nakarai Chemicals Co. (Kyoto, Japan). They were converted into the pyrophosphate derivatives by the method described previously.³⁾ Free fatty acids, acetyl-CoA, and palmitoyl-CoA were purchased from Sigma (St. Louis, MO).

Results

Purification of HB-PP transferase

Solubilized 4HB-PP transferase was partially purified by DEAE-Sepharose CL-6B and Sephacryl S-400 column chromatography (Table I). The specific activity of the purified enzyme was 3.4-fold higher than the crude preparation. Since the partially purified enzyme was not very stable, the purification procedures had to be done within two days. As shown in Fig. 1 the enzyme was eluted in a single but rather broad peak from Sephacryl S-400 column. The fractions showing highest activity (No. 21–23) were used for the following experiment. Treatments with ammonium sulfate or ethanol easily inactivated the enzyme activity (data not shown).

Table I. PURIFICATION STEPS OF 4HB-PP TRANSFERASE

Preparation	Volume (ml)	Total protein (mg)	Total activity ($\times 10^5$ cpm) (%)	Specific activity ($\times 10^5$ cpm/mg) (ratio)
Crude enzyme	60 (57) ^a	96.0	156 (100)	1.6 (1.0)
DEAE-Sepharose	3 (2) ^a	3.9	15 (9.6)	3.8 (2.4)
CL-6B, PEG ppt				
Sephacryl S-400	12	0.48	2.6 (1.7)	5.4 (3.4)

^a Volume used in the next step.

Enzyme activity was assayed in the presence of 1.4 mM bacterial phospholipid for all preparations.

Fig. 1. Elution Profile of the Column Chromatography of Sephacryl S-400 for the Purification of 4HB-PP Transferase.

The eluates were estimated for the protein contents with 0.2 ml out of each fraction (4 ml) by Bradford's method,⁴⁾ and assayed for the 4HB-PP transferase activity with another 0.2-ml sample that was mixed with 1.4 mM of the bacterial phospholipids, by the method described in Materials and Methods. The three fractions indicated with an arrow were used as enzyme solution in the following experiments.

Effects of phospholipid on the purified enzyme

Cellular phospholipid activated the Triton-solubilized enzyme 3-fold as reported in our preceding paper.³⁾ The effects of added phospholipid were more clearly shown when PEG-treated enzyme preparation was used (Table II). This preparation had a phospholipid content of one-third of that of non-treated crude enzyme (data not shown). Further purified enzyme after Sephacryl S-400 gel filtration absolutely required cellular phospholipid for recovery of the enzyme activity (Table II). These results suggest that phospholipid has an essential role for the function of 4HB-PP transferase.

Specificity to chain length of polyprenyl-PP

Pyrophosphate derivatives of polyprenol with different chain lengths (polyprenyl-PP) were prepared as described in Materials and Methods and enzyme activity to these polyprenyl-PPs was measured. Out of them, hexaprenyl-PP and pentaprenyl-PP, reacted with 4-hydroxy[7-¹⁴C]benzoic acid to a similar extent to nonaprenyl-PP (Table III, Exp. I),

Table II. STIMULATION OF THE ENZYME ACTIVITY BY PHOSPHOLIPID

Enzyme	Phospholipid	Activity (cpm)	Activity (ratio)
Crude enzyme	+	3200	1.00
	—	1040	0.32
PEG precipitate	+	3400	1.00
	—	435	0.13
Purified enzyme	+	3220	1.00
	—	120	0.04

Crude enzyme; the preparation solubilized from membrane with Triton X-100, PEG precipitate: enzyme after polyethylene glycol 6000 treatment of crude enzyme, Purified enzyme; the preparation after Sephacryl S-400 column. Phospholipid was prepared from *P. putida* as described in Materials and Methods and used with 1.4 mM concentration. The amount of enzymes of each preparation was changed as the enzyme activity showed around 10³ cpm counts when phospholipid was added.

but tetraprenyl-PP and farnesyl-PP did not react (Table III, Exp. II). These results demonstrated that the substrate specificity for polyprenyl-PP was not very strict, but at least more than 4 isoprene units of chain length were necessary.

Table III. SUBSTRATE SPECIFICITY OF THE TRANSFERASE TO POLYPRENYL-PP

Substrate (chain length)	Concentration (μ M)	Activity (cpm) (%)
Exp. I		
Nonaprenyl-pp (9 units)	50	1400 (100)
Hexaprenyl-PP (6 units)	20	950 (68)
Pentaprenyl-PP (5 units)	50	1200 (86)
Exp. II		
Nonaprenyl-pp (9 units)	30	1610 (100)
Tetraprenyl-PP (4 units)	60	100 (6)
Farnesyl-PP (3 units)	60	10 (1)
Phytol-PP (4 units, saturated)	30	10 (1)

The reaction mixture contained 0.1 ml of enzyme solution (4 μ g protein) after Sephacryl S-400 and 1.4 mM bacterial phospholipid, and each polyprenyl-PP as substrate at the indicated concentration. The activity was shown by total counts per tube.

Table IV. EFFECTS OF UBIQUINONES OR MENAQUINONE ON THE ACTIVITY OF 4HB-PP TRANSFERASE

Quinones ^a	Enzyme activity (cpm) (%)
—	1630 (100)
UQ ₁₀	1610 (99)
UQ ₉	1640 (101)
UQ ₈	1820 (112)
MK ₉	1740 (107)

^a Added in the final concentration of 1 mM.

Abbreviations of quinones are described in Materials and Methods.

The amount of enzyme and phospholipid used in the experiment were as same as described in Table III.

Effects of end-product and the related compounds

The effects of ubiquinones on the enzyme activity were examined. As shown in Table IV, addition of 1 mM of UQ₉ (end-product of the biosynthetic pathway in the bacteria), or ubiquinones with different chain length had no inhibitory effect on the enzyme activity. Neither did MK₉ inhibit the activity.

Inhibition by the lipid metabolites

In preliminary experiments with crude enzyme, it was found that the activity was inhibited by the addition of free fatty acids.

Table V. EFFECTS OF LIPID METABOLITES ON THE ACTIVITY OF 4HB-PP TRANSFERASE

Effectors ^a	Enzyme activity (cpm) (%)
—	1630 (100)
Acetyl-CoA	1450 (89)
Isopentenyl-PP	370 (23)
Isopentenol	1710 (105)
Palmitoyl-CoA	90 (6)
<i>cis</i> -Vaccenic acid	310 (19)

^a Added in final concentration of 1 mM.

The amount of enzyme and phospholipid were as same as described in Table III.

The inhibition was confirmed with the purified enzyme. One mM *cis*-vaccenic acid inhibited about 80% of the enzyme activity (Table V). The intermediate of fatty acid biosynthesis, acyl-coenzyme A (–CoA) inhibited the enzyme more severely than free fatty acids. As shown in Table V, palmitoyl-CoA nearly completely inhibited the activity. In contrast, acetyl-CoA inhibited only 11% of the enzyme activity, indicating that a long chain form of acyl-CoA was essential for the strong inhibitory effect. The other precursor of isoprenoid biosynthesis, isopentenyl-PP, and isopentenol were also examined. Isopentenol had no effect, unlike isopentenyl-PP, which inhibited about 80% of the enzyme activity.

Discussion

4HB-PP transferase was thought to be a key enzyme of ubiquinone biosynthesis,²⁾ but few biochemical studies have been done, especially on the enzyme of prokaryotes, because of the difficulty of purification of the enzyme. In this paper, we did attempt to purify 4HB-PP transferase from *P. putida*, and succeeded in partial purification of the enzyme using ion exchange column chromatography and gel filtration after solubilization with Triton X-100. This preparation made it possible to study the transferase more precisely and to investigate the enzyme as a possible regulatory step in ubiquinone biosynthesis.

By using the purified enzyme, it was clearly

shown, in the first place, that phospholipid was an essential factor for the transferase activity. It is interesting to find whether the phospholipid is required as an activator in the catalytic mechanism or as a factor for stabilizing the enzyme structure. We think that the phospholipids stabilize the transferase, because a considerable amount of cellular phospholipids is coprecipitated with the enzyme (unpublished data), and the enzyme tends to become unstable when phospholipids are removed by further treatment.

Secondly, it was found that this enzyme was not essential in deciding the proper side chain precursor of the polyprenylquinone. Bacterial species as well as higher organisms harbor an ubiquinone type with a well determined chain length of isoprenoid, and thereby this characteristic has been used for bacterial taxonomy.⁸⁾ Therefore, we first assumed that 4HB-PP transferase had a strict substrate specificity for the chain length of polyprenyl-PP, but the result of our study was different. Similar results were observed in early studies using the membrane fraction of mitochondria⁹⁾ and cell homogenate of *E. coli*.¹⁰⁾ These results demonstrate that 4HB-PP transferase does not decide the length of the isoprenoid side chain in the ubiquinone biosynthesis, but a polyprenyl-PP synthetase system indirectly would control the chain length. The enzyme, however, was shown to reject the prenyl-PP homologues shorter than pentaprenyl-PP. This property should be important to cells for protection against binding farnesyl-PP and tetraprenyl (geranylgeranyl)-PP, which are probably more abundant isoprenoid intermediates in the cells.

Further, 4HB-PP transferase was inhibited by various lipids such as free fatty acids, their

CoA derivatives, and isopentenyl-PP. These results suggest that the enzyme has indeed an important regulatory role in overall ubiquinone biosynthesis. Cell growth and cell membrane biogenesis are usually reduced under stringent conditions so that this may cause accumulation of such lipid intermediates in the cell. These metabolites should behave as negative effectors to the transferase, probably as well as to other membrane-related biosynthetic enzymes. Another possibility is that the enzyme makes a stable micelle-like structure with cellular phospholipids, which is easily disrupted by longer fatty acids and their CoA derivatives.

We believe that this and the preceding papers shed light on several important roles and properties of 4-hydroxybenzoate-polyprenyltransferase in ubiquinone biosynthesis.

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