**Studies on the Biosynthesis of Bialaphos. Biochemical Mechanism of C–P Bond Formation:
Discovery of Phosphonopyruvate Decarboxylase which Catalyzes the Formation
of Phosphonoacetaldehyde from Phosphonopyruvate[†]**

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The biosynthetic step following the phosphoenolpyruvate (PEP) phosphomutase reaction which forms a C–P bond of bialaphos was proven by the identification of phosphonopyruvate (PnPy) and phosphonoacetaldehyde (PnAA) as intermediates in the culture broth of *Streptomyces hygroscopicus*, a producing organism of bialaphos, and by detection of enzymatic decarboxylation of PnPy to PnAA. Purified PnPy decarboxylase turned out to require thiamine diphosphate and Mg²⁺ as cofactors. PnPy decarboxylase drives the unfavorable forward reaction to form PnPy catalyzed by PEP phosphomutase and is suggested to be essential to C–P compound biosynthesis.

Among vast number of secondary metabolites, bialaphos (BA), a product of *Streptomyces hygroscopicus* SF-1293 with a herbicidal activity^{1,2)} is characterized as a tripeptide possessing a unique C–P–C bond. Through the extensive studies in the past several years, we have revealed that the biosynthetic pathway of BA consists of more than 13 steps^{3~15)}. Among them, the first C–P bond forming reaction has turned out to be seemingly common to the biosyntheses of all the C–P compounds.

Since the discovery of the first natural C–P compound, 2-aminoethylphosphonic acid (AEP)¹⁶⁾, the biosynthetic mechanism of the C–P bond formation has attracted considerable interest, and the enzyme phosphoenolpyruvate phosphomutase (PEP phosphomutase) catalyzing the formation of phosphonopyruvate (PnPy) from phosphoenolpyruvate (PEP) (Fig. 1) has been purified and identified from several organisms^{6,17~19)}. This enzyme had remained as a big mystery for more than three decades, since no one succeeded in detecting its activity even in cell free systems.

However, our recent finding that the equilibrium of the C–P bond formation reaction favors strongly the formation of phosphate ester, *i.e.* cleavage of the C–P bond to give phosphoenolpyruvate^{17,18,20)} enabled purification of the enzyme by observing this reverse reaction. This characteristic feature of PEP phosphomutase im-

plied that a mechanism to convert effectively PnPy to the next intermediate would be essential to produce C–P compounds.

The next step following the PEP phosphomutase reaction, which is considered to be decarboxylation of PnPy to generate phosphonoacetaldehyde (PnAA) (Fig. 1), has recently been the focus of our research interest. The first identification of PnPy had been as a product of bio-conversion from PEP using the cell free extract of *Tetrahymena pyriformis* which produced AEP²¹⁾. Recently PnPy was confirmed to be the substrate of PEP phosphomutase^{4,19)}. However, PnAA and even PnPy were still proposed intermediates to be identified as natural compounds, and the reactions including these compounds following PEP phosphomutase reaction were still unclear. We have attempted to investigate the decarboxylation of PnPy (step 2 reaction) to make clear the mechanism of C–P bond formation.

In the course of our study, various kinds of BA non-producing blocked mutants of *S. hygroscopicus* had been identified and characterized^{5~8,11,12)}. One of these blocked mutants, *S. hygroscopicus* E26, is defective in PEP phosphomutase and does not produce any C–P compounds⁴⁾. Another BA non-producing mutant, *S. hygroscopicus* NP46, accumulates hydroxyethylphosphonic acid (HEP) in the culture broth²²⁾, probably because it is

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Fig. 1. Proposed biosynthetic pathway of bialaphos.

PEP, phosphoenolpyruvate; PnPy, phosphonopyruvate; PnAA, phosphonoacetaldehyde; HEP, hydroxyethylphosphonic acid; HMP, hydroxymethylphosphonic acid.

defective in the reaction following PnAA generation (Fig. 1). In addition, several C-P compound-producing microorganisms were used in our study in order to facilitate to analyze the step 2 reaction in detail by exploiting their characteristic biochemical features. *Pseudomonas gladioli* B-1 was identified as an HEP producing strain, and PEP phosphomutase was purified from this strain¹⁸⁾. *Streptomyces wedmorensis* 144-91 is a high-producer of fosfomycin and its mutant NP-7 was suggested to be a step 2 deficient mutant by bio-conversion experiment²³⁾. *Pseudomonas viridiflava*, identified as a fosfomycin and fosfadecin producing strain²⁴⁾, was also utilized in this experiment.

Here we report the determination of the mechanism of step 2 by identification of proposed intermediates, PnPy and PnAA, in the culture broth and by discovery of the enzymatic activity of PnPy decarboxylase which catalyzes the conversion of PnPy to PnAA. We also describe the purification and partial characterization of PnPy decarboxylase.

Materials and Methods

Materials

DEAE-cellulose DE52 was purchased from Whatman Ltd., and Mono Q HR 5/5 and PD-10 were obtained from Pharmacia LKB Biotechnology. TSK-gel Phenyl-5PW and TSK-gel G4000SW were purchased from Toso

Co. SDS-polyacrylamide gradient gel was purchased from Daiichi Pure Chemical Co., Ltd.

HPLC was performed by using an 800 series HPLC system (Japan Spectroscopic Co., Ltd.).

PnPy was prepared by non-enzymatic transamination²⁵⁾ from phosphonoalanine (PnAla), a product of Sigma Chemical Co. PnAA was prepared as described by ISBEL²⁶⁾.

All other chemicals without specification were of reagent grade and were purchased from Nacalai Tesque Inc.

Bacterial Strains and Growth Conditions

S. hygroscopicus E26, NP46 and *Streptomyces wedmorensis* 144-91 and NP-7 were obtained from the Meiji Seika Kaisha Culture Collection. *Pseudomonas viridiflava* was obtained from Takeda Chemical Industries, Ltd.

Cultures of *Streptomyces* were routinely stored at -20°C in 30% glycerated S-1 medium consisting of 2% soluble starch, 1% polypeptone, 0.3% meat extract and 0.05% K_2HPO_4 . Cells from frozen culture (0.4 ml) were inoculated into a tube containing 10 ml of S-1 medium and cultured at 27°C for 2 days with shaking. This seed culture (1.2 ml) was inoculated into the 60 ml BA production medium contained in a 500-ml Erlenmeyer flask and incubated at 27°C for 4 or 5 days with shaking. BA production medium contained 7% glucose, 3.9% wheat germ, 2.5% sun grain (Sungross; Suntory Ltd.), 0.1% KH_2PO_4 , 0.0001% CoCl_2 and silicon KM-72 antifoam (Shin-etsu Chemical Co.), and pH was adjusted to 7.0.

³¹P NMR Analysis of Broth Filtrates

Broth filtrates were concentrated 10 times and analyzed by a JEOL GSX500 NMR spectrometer operating at 202.35 MHz at 25°C. ³¹P-Chemical shift values are reported relative to external 85% H₃PO₄.

For dinitrophenylhydrazine (DNP) modification experiments, broth filtrate (30 ml) was mixed with 3 ml of 0.5% DNP in 2 N HCl and stored at 4°C for 30 minutes. The reaction mixture was extracted with ethyl acetate, concentrated *in vacuo*, and then the residue was dissolved in ethyl acetate for ³¹P NMR analysis.

Enzyme Assay

The reaction mixture (2 ml) consisting of 0.5 mM PnPy, 1 mM thiamine diphosphate and 5 mM MgSO₄ in the cell free extracts was incubated at 30°C with shaking and the reaction was terminated by addition of 250 µl of 0.5% DNP in 2 N HCl. The DNP modified reaction products were extracted with ethyl acetate and dried with N₂ gas. This sample was dissolved in 100 µl of 10% acetonitrile in 4% phosphoric acid and applied to HPLC analysis using a TSK-gel ODS 80 column (7.6 × 250 mm, Toso Co.). After washing with 10% acetonitrile in 4% phosphoric acid for 5 minutes, the elution was carried out at a flow rate of 0.6 ml/minute with a linear gradient of 30 to 35% acetonitrile in 4% phosphoric acid in 30 minutes. DNP modified compounds were detected by the absorbance at 365 nm.

Preparation of Cell Free Extract

Mycelia of *S. hygroscopicus* mutants, *S. wedmorensis* and cells of *P. viridiflava* were harvested by centrifugation (3,000 × *g*, 15 minutes, 4°C) and were suspended in 3-fold volumes of 50 mM Tris-HCl buffer (pH 7.5) after washing twice with the same buffer. The suspension was subjected to ultrasonic treatment for 10 minutes (2N-100; Toyo Rikoh Co.) Cell debris was removed by centrifugation at 14,000 × *g* and 4°C for 15 minutes.

Purification

The cell free extract was prepared as above from 100 g cell paste, which was harvested from 480 ml culture broth. All subsequent steps were performed at 4°C. Protein concentration was determined by using Protein Assay (Bio-Rad). The cell free extract (300 ml) was brought to 30% saturation by addition of solid ammonium sulfate over 30 minutes, stirred for an additional 30 minutes, and centrifuged (14,000 × *g*, 15 minutes). The supernatant was collected and adjusted to 60% saturation by addition of solid ammonium sulfate over 30 minutes, stirred for an additional 30 minutes, and centrifuged. The resulting pellet was dissolved in 2,000 ml of 50 mM Tris-HCl buffer (pH 7.5) and was loaded on a DEAE cellulose column (60 by 300 mm) previously equilibrated with the same buffer. After washing with the same buffer, the column was eluted with a linear gradient from 0 to 0.3 M NaCl in the same buffer in a volume of 1,400 ml. Fractions of 10 ml each were collected. PnPy decarboxylase was eluted

at around 0.05 M NaCl concentration.

The active fractions eluted at 0.05 to 0.08 M NaCl were combined and precipitated by addition of solid ammonium sulfate to 60% saturation. After centrifugation, the pellet was dissolved in sodium phosphate buffer (50 mM, pH 7.0) containing 1.2 M ammonium sulfate. The sample was loaded on a hydrophobic interaction column, TSK-gel Phenyl-5PW (21.5 by 150 mm), previously equilibrated with the same buffer, running on an HPLC system at a flow rate of 3 ml/minute. The elution was carried out with a linear gradient from 0.72 to 0 M ammonium sulfate in sodium phosphate buffer (50 mM, pH 7.0) after washing with 0.72 M ammonium sulfate in the same buffer. PnPy decarboxylase was eluted at an ammonium sulfate concentration of around 0.65 M.

The active fractions were pooled and concentrated by ultrafiltration using Centricon (Amicon) and applied to gel filtration by an HPLC system using a TSK-gel G3000SW column. The gel filtration was performed with sodium phosphate buffer (10 mM, pH 7.0) containing 0.1 M NaCl and 10% glycerol at a flow rate of 0.4 ml/minute. Fractions of 0.8 ml each were collected.

The pooled enzyme solution was finally loaded at a flow rate of 1.0 ml/minute on a Mono Q HR 5/5 column equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The elution was carried out with a linear gradient from 0 to 0.15 M NaCl in the same buffer, and each peak was collected separately by monitoring the absorbance at 280 nm. Purified PnPy decarboxylase was eluted at around 0.05 M NaCl.

PEP Phosphomutase Coupling Reaction

The reaction mixture (2 ml) contained 1.5 mM thiamine diphosphate, 5 mM Mg²⁺, 50 µl of PnPy decarboxylase preparation (0.1 mg protein), and various concentrations of PEP and PEP phosphomutase in 50 mM Tris-HCl buffer (pH 7.5). PEP phosphomutase (60 ng/µl) was purified from *P. gladioli* B-1, and PnPy decarboxylase was partially purified from *S. hygroscopicus* E26 by ammonium sulfate precipitation (30~60%). The concentrations of PEP were 1, 10, 50 and 100 mM. The concentration of PEP phosphomutase was increased to 150 ng/ml when the PEP concentration was 50 mM. The reaction was initiated by addition of PnPy decarboxylase. The reaction mixture was incubated at 30°C for 7 hours with shaking and terminated by DNP treatment under acidic condition. DNP derivatives were extracted with ethyl acetate and evaporated, and the residues were subjected to HPLC analysis.

Results

Determination of the Step 2 Reaction

³¹P NMR spectroscopy is a very suitable method for detecting selectively C-P compounds from the other phosphorous compounds even in crude samples, although the detection sensitivity is not so high²⁷⁾. Thus an attempt

was made to detect C-P compounds accumulated in the culture broths of various mutants of *S. hygroscopicus* using ^{31}P NMR. This experiment proved the accumulation of several C-P compounds such as AEP, hydroxymethylphosphonic acid (HMP), HEP²²⁾ and phosphonoalanine (PnAla) which had been isolated from several blocked mutants of the BA producer¹¹⁾, but PnPy and PnAA were not seen, presumably due to their instability or their low concentrations in the culture broth. Both PnPy and PnAA are considerably unstable, while the C-P bonds of other C-P compounds are very stable. PnPy easily decomposed to pyruvic acid and phosphoric acid, and PnAA to acetaldehyde and phosphoric acid. Since only PnPy and PnAA with a carbonyl group were assumed to be involved in the early stage of the C-P compounds biosynthesis, we tried to detect them after modification with dinitrophenylhydrazine (DNP). ^{31}P NMR analysis of a DNP-treated broth filtrate of NP46, a blocked mutant of *S. hygroscopicus* accumulating HEP in the culture broth²⁴⁾, showed distinct signals of PnPy and PnAA derivatives at 21.5 and 19.5 ppm, respectively (Fig. 2). The signals of PnPy and PnAA were not seen in the mutant E-26 defective in step 1. These

Fig. 2. ^{31}P NMR spectrum of DNP-treated broth filtrate of *S. hygroscopicus* NP46.

Table 1. Conversion of C-P compounds to bialaphos by mutant strains of *S. hygroscopicus*.

C-P compound*	Bialaphos production ($\mu\text{g/ml}$)	
	E26	NP46
PnPy	3.0	-
PnAla	13.0	-
PnAA	0.2	-
AEP	2.5	-
HMP	5.5	15.0

Bialaphos production was measured by antimicrobial activity against *Bacillus subtilis*.

* Concentration of substrate; 100 $\mu\text{g/ml}$.

results indicated that PnPy and PnAA were produced by *S. hygroscopicus* in the early stage of the BA biosynthesis.

Conversion experiments of PnPy, PnAA and other C-P compounds with washed cells of several BA non-producing mutants were performed as previously reported¹¹⁾. The results showed that PnPy, PnAla (a biological equivalent of PnPy) and PnAA were converted to BA by the strain E26 but neither were converted by NP46 (Table 1). On the other hand the next intermediate, HMP was converted to BA by both strains of E26 and NP46. From the results of these conversion experiments using washed cells as well as their presence in the broth filtrate, PnPy and PnAA were confirmed as biosynthetic intermediates of BA.

PnPy Decarboxylase Activity

In order to analyze the step 2 pathway from PnPy to PnAA in detail, the enzymatic activity of step 2 was examined with the cell free extract of NP46. Conversion of PnPy to PnAA was detected by a reverse phased HPLC analysis system after the produced PnAA was treated with DNP. The retention times of PnPy-DNP and PnAA-DNP in this assay were 21.5 and 25.5 minutes, respectively. By this method PnAA-DNP was detected in the reaction mixture using the NP-46 cell-free extract only when incubation was done for 4 to 6 hours (Fig. 3), despite the unfavorable presence in the reaction mixture of PEP phosphomutase which should disturb the enzyme

Fig. 3. HPLC chromatogram of PnPy decarboxylase assay.

The enzymatic reaction and sample preparation for HPLC were performed as described in materials and methods, and 50 μl of the sample was analyzed by HPLC.

assay by consuming PnPy. PnAA seemed to be gradually decomposed or converted to other compounds after being produced from PnPy. When a heated cell free extract was used for this assay, PnAA-DNP was not produced. Therefore, this assay system enabled us to detect the activity of the novel enzyme, PnPy decarboxylase. The enzymatic activity was also detected in the cell free extract of the mutant E-26 lacking the PEP phosphomutase activity.

These results indicated that step 2 reaction of the BA biosynthesis following the PEP phosphomutase reaction is the decarboxylation of PnPy to generate PnAA catalyzed by PnPy decarboxylase. Thus the first two steps of the BA biosynthesis were identified definitely.

Purification of PnPy Decarboxylase

To purify PnPy decarboxylase, *S. hygroscopicus* E26 lacking PEP phosphomutase activity was utilized as an enzyme source, because the presence of PEP phosphomutase disturbed the detection of the enzymatic activity of the decarboxylase during purification. The specific activity of this enzyme could not be accurately estimated by the assay method described in Materials and Methods due to the gradual decomposition of PnAA during the reaction. From the cell free extract containing 2,187 mg protein PnPy decarboxylase was purified to homogeneity (*ca.* 0.36 mg) by a five-step protocol as described in Materials and Methods.

Properties of PnPy Decarboxylase

The molecular weight of the PnPy decarboxylase was estimated as 135 kDa by gel filtration using TSK-gel G3000SW and its subunit was estimated as 36 kDa by SDS-PAGE. This enzyme was stable for 3 weeks during storage in 50 mM Tris-HCl buffer (pH 7.5) at 4°C. Pyruvate decarboxylase catalyzing the similar reaction requires thiamine diphosphate (TPP) and Mg^{2+} as cofactors. Requirement of these cofactors for the activity was examined with an enzyme preparation partially purified by ammonium sulfate fractionation (30~60%) followed by desalting by PD-10 column chromatography. The PnPy decarboxylase activity was detected in the reaction mixture containing both TPP and Mg^{2+} but not in those lacking either or both of TPP and Mg^{2+} , indicating that PnPy decarboxylase required TPP and Mg^{2+} as cofactors. Pyruvate decarboxylase^{28,29)} also catalyzes α -keto acid decarboxylation and requires TPP as a cofactor. Pyruvate decarboxylase, however, could not utilize PnPy as a substrate for decarboxylation (data not shown).

Table 2. Generation of PnAA *via* PnPy from PEP by PEP phosphomutase and PnPy decarboxylase^a coupling system.

PEP (mM)	PEP phosphomutase (ng)	PnPy (μ M)	PnAA (μ M)
1	180	ND ^b	ND ^b
10	180	36	27
50	0	0	0
50	180	81	25
50	300	133	51
100	180	171	24

^a PnPy decarboxylase was prepared from the cell free extract of *S. hygroscopicus* E26 by ammonium sulfate precipitation (30~60%).

^b The peaks of these compounds were too faint to estimate.

Synthesis of PnAA by Biological System

In order to confirm the C-P bond formation pathway from step 1 to step 2, a PEP phosphomutase-PnPy decarboxylase coupled system was tested for the production of PnAA from PEP. PnPy decarboxylase was prepared from *S. hygroscopicus* E26 to avoid contamination of PEP phosphomutase activity. PEP phosphomutase purified from *P. gladioli* B-1 was used for this system, because PEP phosphomutase of *S. hygroscopicus* was too unstable to be purified enough to avoid the contamination of PnPy decarboxylase. Since the ratio of the substrate (PEP) to the product (PnPy) was reported to be over 500:1 at the equilibrium of the PEP phosphomutase reaction, a large excess amount of the substrate PEP up to 100 mM was used in this reaction. Neither PnPy nor PnAA were produced from PEP at the concentration of 50 mM in the presence of only PnPy decarboxylase (Table 2). By addition of *P. gladioli* B-1 PEP phosphomutase into the reaction mixture, PnAA was produced from PEP *via* PnPy by this coupled system even when the concentration of PEP was as low as 10 mM. This suggested that both enzymes functioned in C-P compound biosynthesis and that step 2 reaction converted PnPy to PnAA, driving the step 1 reaction in the forward direction.

PnPy Decarboxylase Activity of Other C-P Compound Producing Strains

In the reaction mixture containing the cell free extract of a fosfomycin producing strain, *S. wedmorensis* 144-91, most of the substrate PnPy was converted to PnAA (Fig. 4A), indicating that this strain possesses strong PnPy decarboxylase activity. On the other hand PnAA was not

Fig. 4. PnPy decarboxylase activities of other C-P compound producing strains.

Arrowheads indicate the peaks of PnAA-DNP.

A, Fosfomycin producing strain *S. wedmorensis* 144-91; B, step 2 deficient mutant *S. wedmorensis* NP-7; C, fosfadecin producing strain *Pseudomonas viridiflava*.

detected in the reaction mixture using the cell free extract of *S. wedmorensis* NP-7 (Fig. 4B), supporting biochemically that NP-7 is a step 2 deficient mutant. PnAA was also detected when the cell free extract of *P. viridiflava* was used in the reaction mixture (Fig. 4C), indicating the existence of PnPy decarboxylase in a Gram-negative bacterium producing a C-P compound, fosfadecin. These indicated that the step 2 reaction is common to all of C-P compound biosyntheses and catalyzed by PnPy decarboxylase.

Discussion

PnPy and PnAA were confirmed as biosynthetic intermediates for BA by the work reported herein. The discovery of PnPy decarboxylase as well as the coupling system consisting of PEP phosphomutase and PnPy decarboxylase indicated that step 2 reaction is common to the biosyntheses of all C-P compounds. This was also supported by the detection of PnPy decarboxylase activities in *S. wedmorensis* and *P. viridiflava* which produce fosfomycin and fosfadecin, respectively.

The non-oxidative α -keto acid decarboxylation of PnPy is analogous to the pyruvate decarboxylase reaction and needs TPP and Mg^{2+} as cofactors. Pyruvate decarboxylase, however, did not utilize PnPy as a substrate despite its wide substrate specificity, excluding the possibility that the enzyme plays a role in the biosynthesis of BA. Thus PnPy decarboxylase is concluded to be the specific enzyme catalyzing the conversion of PnPy to PnAA in BA biosynthesis.

This study showed that PnAA was produced from PEP by the coupled system of PEP phosphomutase and PnPy decarboxylase. Although the accurate conversion ratio could not be estimated due to gradual decomposition of PnAA during the reaction, step 2 reaction proved to drive the equilibrium of the step 1 reaction, which otherwise favored the formation of PEP, in the forward direction in C-P compound biosynthesis.

In living systems PnPy may well be converted to PnAla which is subjected to several different metabolisms. Our studies, however, suggest that most C-P compounds are synthesized through PnPy and PnAA, and that PnPy decarboxylase is essential to C-P compound biosynthesis. PnAA is converted to 2-hydroxypropylphosphonic acid in fosfomycin biosynthesis³⁰⁾ and is believed to be converted to HMP in BA biosynthesis²²⁾. PnAA is located at the pivotal point from where biosynthetic pathways diversify to form various C-P compounds and the next reactions following PnPy decarboxylation would stabilize the C-P bonds. Thus PnPy decarboxylase is critical for the structural diversity of C-P compounds as well as for the C-P bond formation in the C-P compound biosyntheses. Further studies on the reaction mechanism and the enzymatic properties of this enzyme are necessary to investigate the significance of C-P compound metabolism in living system.

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