Purification and Characterization of Phosphoenolpyruvate Phosphomutase from *Pseudomonas gladioli* B-1

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Phosphoenolpyruvate phosphomutase (PEPPM) catalyzes C-P bond formation by intramolecular rearrangement of phosphoenolpyruvate to phosphonopyruvate (PnPy). We purified PEPPM from a gram-negative bacterium, *Pseudomonas gladioli* B, isolated as a C-P compound producer. The equilibrium of this reaction favors the formation of the phosphate ester by cleaving the C-P bond of PnPy, but the C-P bond-forming reaction is physiologically significant. The C-P bond-forming activity of PEPPM was confirmed with a purified protein. The molecular mass of the native enzyme was estimated to be 263 and 220 kDa by gel filtration and polyacrylamide gel electrophoresis, respectively. A subunit molecular mass of 61 kDa was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, indicating that the native protein was a tetramer. The optimum pH and temperature were 7.5 to 8.0 and 40°C, respectively. The K_m value for PnPy was 19 ± 3.5 μM, and the maximum initial velocity of the conversion of PnPy to phosphoenolpyruvate was 200 μM/s/mg. PEPPM was activated by the presence of the divalent metal ion, and the K_m values were 3.5 ± 1.4 μM for Mg^{2+}, 16 ± 5 nM for Mn^{2+}, 3.0 ± 1.5 μM for Zn^{2+}, and 1.2 ± 0.2 μM for Co^{2+}.

Since the discovery of the first natural C-P compound, 2-aminoethylphosphonic acid (10), various metabolites with C-P bonds have been isolated and the presence of phosphonates and phosphinates in a variety of biological systems has been established. Extensive studies on their unique C-P bond formation mechanisms (1, 8, 9, 20, 23, 24) indicated that the first step of the C-P bond formation pathway is the intramolecular rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate (PnPy) and that the equilibrium of this reaction heavily favors the formation of the phosphate ester (Fig. 1) (20). The enzyme catalyzing this reaction, phosphoenolpyruvate phosphomutase (PEPPM), was purified from *Tetrahymena pyriformis* by Harvard (20) and Maryland groups (2) and partially from *Streptomyces hygroscopicus* by us (8). Although other enzymes catalyzing C-P bond-forming reactions, carboxyphosphonoenolpyruvate phosphonomutase (6) and P-methylation enzyme (14), were detected and isolated by us, they catalyzed the reactions specific for bialaphos biosynthesis (7, 12). On the other hand, PEPPM converts a ubiquitous metabolite, PEP, to a C-P compound and is assumed to be common to all of the C-P compound-producing organisms.

Although the presence of C-P compounds extends widely from bacteria to higher animals, the ability to produce C-P compounds is limited to lower organisms, such as bacteria, coelenterates (18), and mollusks (13). Many C-P compounds with various structural features have been identified as metabolites of bacteria, especially *Actinomycetes* species. Therefore, various microorganisms are expected to produce C-P compounds and to possess PEPPM. So far, purification of PEPPM and detection of its activity have been performed only with *Actinomycetes* species, except for *T. pyriformis* (8). To clarify the formation mechanisms of the C-P bond in more detail, PEPPM from other organisms needs to be investigated. In a previous paper, we reported that C-P compound-producing strains were obtained by screening soil samples from exploiting the nature of PEPPM, which is

favoring the cleavage of the C-P bond of PnPy to form PEP (17). One of these strains, *Pseudomonas gladioli* B-1, produced hydroxylethylphosphonic acid, known as a biosynthetic intermediate of fosfomycin (11), and PEPPM activity was detected in the cell extract of this strain. We report herein the purification and characterization of PEPPM from *P. gladioli* B-1.

MATERIALS AND METHODS

Materials. DEAE-cellulose DE52 was purchased from Whatman, Ltd., and Mono Q HR 5/5 and Phenyl-Superose HR 5/5 were obtained from Pharmacia LKB Biotechnology, Inc. TSKgel Phenyl-5PW and TSKgel G4000SW were purchased from Toso Co., and the hydroxyapatite column (7.5 by 100 mm) was a product of Toa Nenryo Kogyo Co.

High-performance liquid chromatography (HPLC) was performed by using an 800 series HPLC system (Japan Spectroscopic Co., Ltd.).

Native and sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels were purchased from Daiichi Pure Chemical Co., Ltd.

PnPy was prepared by transamination of 2-amino-3-phosphonopropionic acid (3), a product of Sigma Chemical Co. All other chemicals were of reagent grade and were purchased from Nacalai Tesque, Inc.

Organisms and culture conditions. The organism used in this study was *P. gladioli* B-1, which had been isolated from a soil sample by screening and which possessed the ability to cleave the C-P bond of 2-amino-3-phosphonopropionic acid (16, 17).

P. gladioli B-1 was grown in a 50-liter jar fermentor containing a medium with the following composition (per liter): 10 g of glucose, 0.5 g of L-asparagine, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $FeSO_4 \cdot 7H_2O$. Cells were grown at pH 7 and 27°C with aeration of 15 liters/min. From 12 h after the initiation of the culture, the activity of PEPPM was monitored by sampling the cells every 20 min. When the specific activity reached its highest level, the reaction was stopped, the broth was immediately

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\begin{array}{c} CH_2 \\ \| \\ C-O-PO_3H_2 \\ | \\ COOH \end{array}
&
\xrightarrow{\hspace{1cm}}
&
\begin{array}{c} PO_3H_2 \\ | \\ CH_2 \\ | \\ C=O \\ | \\ COOH \end{array}
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\xrightarrow{\;\;CO_2\;\;}
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\\[1em]
PEP & & PnPy \qquad\qquad PnAA
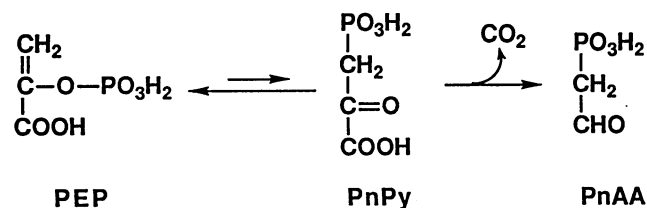\end{array}
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FIG. 1. Proposed biosynthetic pathway for C-P bond formation. PnAA, phosphonoacetaldehyde.

cooled, and the pH was adjusted to 7 with aqueous NaOH. The cells of *P. gladioli* were harvested by centrifugation and washed with 50 mM Tris-HCl buffer (pH 7).

Enzyme assays. The activity of the C-P bond-cleaving reaction catalyzed by PEPPM was assayed with a system consisting of 50 mM Tris-HCl buffer (pH 7.5), 0.27 mM PnPy, 0.15 mM NADH, 0.72 mM ADP, 5 mM $MgSO_4 \cdot 7H_2O$, 5 U of lactate dehydrogenase per ml, and 5 U of pyruvate kinase per ml. The reaction was started by the addition of enzyme preparations, and then the decrease in A_{340} was recorded at 30°C. The activity was determined by measuring the difference in the decrease in absorbance when PnPy was present and when it was absent. One unit of PEPPM activity was defined as the conversion of 1 μmol of PnPy to PEP per min.

The activity of the C-P bond-forming reaction was assayed by using a system consisting of 50 mM MES (morpholineethanesulfonic acid)-NaOH buffer (pH 6.5), 10 to 100 mM PEP, 0.15 mM NADH, and 50 U of malate dehydrogenase per ml. The reaction was started by the addition of enzyme samples, and 30 min after the reaction was initiated, the decrease in A_{340} was recorded at 30°C.

Purification. Washed cells (150 g) from 60 liters of the culture broth were suspended in 400 ml of 50 mM Tris-HCl buffer (pH 7.5) and then disrupted by sonication (2N-100; Toyo Rikoh Co.). The cell debris was removed by centrifugation ($14,000 \times g$, 10 min), and the supernatant was used as the cell extract.

The extract was diluted to 1.5 liters with the same buffer and applied to a DEAE-cellulose DE52 column (4 by 50 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5). After the column was washed first with 50 mM Tris-HCl buffer and then with the same buffer containing 0.075 M NaCl, the enzyme was eluted with the same buffer containing 0.15 M NaCl, and each fraction was collected in a 5-ml volume. The active fractions were combined, and after being diluted fourfold with 25 mM Tris-HCl buffer (pH 7.5), the solution was again applied to a DEAE-cellulose DE52 column (1.6 by 30 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5). After being washed first with 50 mM Tris-HCl buffer and then with the same buffer containing 0.09 M NaCl, the enzyme was eluted with the same buffer containing 0.17 M NaCl.

Active fractions were combined, and solid $(NH_4)_2SO_4$ was gradually added to the solution to give 60% saturation. After being stirred for 1 h, the supernatant was removed by centrifugation ($10,000 \times g$, 10 min), and the pellet was dissolved in 5 ml of 50 mM potassium phosphate buffer containing 30% $(NH_4)_2SO_4$.

The enzyme solution was further purified on an HPLC system with a TSKgel Phenyl-5PW column (21.5 by 150 mm). After the enzyme solution was loaded onto the column previously equilibrated with 1.2 M $(NH_4)_2SO_4$-50 mM po-

tassium phosphate buffer (pH 7.0), the elution was carried out with a linear gradient of 1.2 to 0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl buffer (pH 7.0) at a flow rate of 3.5 ml/min, and each fraction was collected in a 3.5-ml volume. The active fractions were combined and concentrated by precipitation at 70% $(NH_4)_2SO_4$ and were then centrifuged.

The pellet was dissolved in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Gel filtration was carried out with a TSKgel G4000SW column and run with 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 10% glycerol at a flow rate of 0.5 ml/min. Fractions of 0.5 ml each were collected.

The pooled enzyme fractions were finally applied to 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 of 0 to 0.5 M NaCl in the same buffer at a flow rate of 1 ml/min, and each peak was collected by monitoring the A_{280}.

Hydroxyapatite column chromatography. An enzyme sample was applied to a hydroxyapatite column (7.5 by 100 mm) which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The elution was carried out with a linear gradient of 10 to 200 mM potassium phosphate buffer at a flow rate of 0.5 ml/min. PEPPM was eluted with potassium phosphate buffer at a concentration of 120 mM.

Reversed-phase silica gel chromatography. Reversed-phase HPLC was performed with an Asahi-pak C8P-50 column (4.6 by 150 mm) (Asahi Chemical Industry Co., Ltd.). The enzyme sample, desalted by ultrafiltration, was applied to the column previously equilibrated with 0.1% trifluoroacetic acid in H_2O. The elution was carried out with a linear gradient of 0 to 100% CH_3CN at a flow rate of 0.5 ml/min. The PEPPM protein was eluted with CH_3CN at a concentration of 40%.

Biochemical characterization. The molecular weight of the denatured PEPPM was estimated by SDS-polyacrylamide gel electrophoresis (PAGE). Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as marker proteins. Gradient gels (10 to 20%) were used and were stained with Coomassie brilliant blue.

The molecular mass of the native enzyme was estimated by PAGE and by gel filtration. PAGE was carried out with gradient gels (4 to 15%). Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin were used as marker proteins, and gels were stained with Coomassie brilliant blue.

Gel filtration was carried out with a TSKgel G4000SW column that had been equilibrated with 10 mM potassium phosphate buffer containing 0.1 M NaCl-10% glycerol. The reference proteins were thyroglobulin, ferritin, catalase, and aldolase (158 kDa).

RESULTS

Purification of PEPPM. Under the culture conditions described in Materials and Methods, the PEPPM activity of *P. gladioli* B-1 increased to its maximum at the early log phase, as previously reported (17). *P. gladioli* B-1 cells were harvested when the specific activity of PEPPM was at a high level and were disrupted by sonication in 50 mM Tris-HCl buffer (pH 7.5). Purification of PEPPM from 150 g of cell paste was carried out in seven steps (Table 1). The first step was chromatography on DEAE-cellulose. Next, the active

TABLE 1. Summary of purification of PEPPM from *P. gladioli*

Step	Total protein (mg)	Total units	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract	12,500	8,700	0.69	100	
DEAE-cellulose I	780	4,100	5.25	47.1	7.6
DEAE-cellulose II	280	3,400	12.1	39.1	17.5
Ammonium sulfate	31.5	1,950	61.9	22.4	97.3
TSKgel Phenyl-5PW	3.4	1,200	353	13.8	511
Gel filtration	0.74	750	1,010	8.6	1,460
Mono Q	0.21	540	2,570	6.2	3,730

fractions were combined and chromatographed on DEAE-cellulose again. Then the ammonium sulfate fraction (30 to 60%) was subjected to TSKgel Phenyl-5PW column chromatography, and the enzyme was eluted with 0.12 M ammonium sulfate. After gel filtration, Mono Q HPLC was utilized as the final step.

Chromatography on the hydroxyapatite column and chromatography on Phenyl-Superose (the elution procedure was the same as with the TSKgel Phenyl-5PW, except that the flow rate was 0.5 ml/min) of the active fraction of Mono Q HPLC showed that the protein preparation obtained after Mono Q HPLC was almost pure. The hydroxyapatite column with a phosphate gradient gave a sharp peak of PEPPM elution, but the activity of the enzyme was considerably less. This finding is in agreement with the results of a study with PEPPM from *T. pyriformis* reported by Bowman et al. (2). Gel electrophoresis under denatured conditions (SDS-PAGE) (Fig. 2B) and reversed-phase silica gel HPLC also confirmed the protein purity. The enzyme was purified 3,700-fold, with an overall yield of 6%.

Molecular size. The molecular mass of the denatured PEPPM was determined by SDS-PAGE (10 to 20% gradient) analysis to be 61 kDa (Fig. 2B). The molecular mass of the native protein was estimated by a TSKgel G4000SW gel filtration technique and a native PAGE (4 to 15% gradient) technique to be 263 and 220 kDa, respectively (Fig. 2A). These data and the reversed-phase silica gel chromatography indicated that the PEPPM of *P. gladioli* is a homotetramer of 61-kDa proteins.
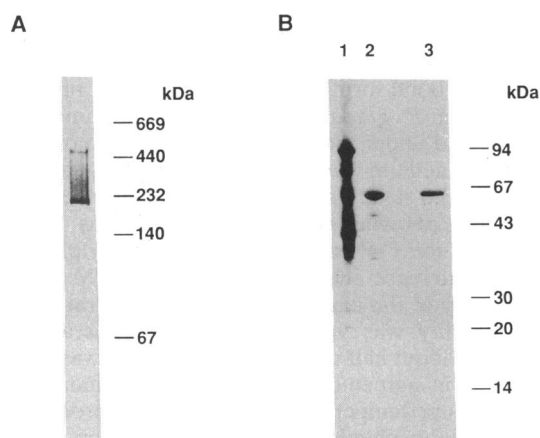

FIG. 2. PAGE analysis of the PEPPM of *P. gladioli*. (A) Native PAGE of purified PEPPM. The native enzyme and an aggregated protein are visible. (B) SDS-PAGE of PEPPM. Lanes: 1, DEAE-cellulose II; 2, TSKgel Phenyl-5PW; 3, Mono Q HPLC.

Stability. The purified enzyme could be stored for more than 1 month at −20°C without significant loss of activity in 50% glycerol solution containing 25 mM potassium phosphate buffer (pH 7).

The stability of the enzyme at various pHs was examined. The enzyme was incubated at 25°C for 30 min in the following buffers (final concentration, 50 mM): CH_3COONa-CH_3COOH (pH 3.4 to 5.9), potassium phosphate (pH 5.3 to 8.0), and Tris-HCl (pH 7.2 to 9.1). Then a sample of the enzyme was taken, and the PEPPM activity was assayed under standard conditions (pH 7.5, 25°C). The enzyme was most stable in the pH range of 6.0 to 8.0, and 50% of its activity was retained even at pH 9.1, but its activity was completely lost at less than pH 5.2.

The stability of the enzyme at various temperatures was examined. After the enzyme had been preincubated for 30 min in 50 mM Tris-HCl buffer (pH 7.5), a sample of the enzyme solution was taken, and the activity was assayed under standard conditions. The enzyme exhibited the following activities at the following temperatures: 70°C, 0%; 60°C, 0%; 50°C, 8%; 45°C, 28.5%; 40°C, 35.7%; 35°C, 49%; 30°C, 98%; and 25°C, 100%.

Activity for forming the C-P bond. The PEPPM was purified as a protein converting PnPy to PEP by using the ADP–pyruvate kinase-NADH–lactate dehydrogenase coupled system as described in Materials and Methods, because the activity to form the C-P bond was undetectable in the crude extract of the cells. For PEPPM from *T. pyriformis* and *S. hygroscopicus*, the enzyme catalyzed the cleavage more than it formed the C-P bond. Thus, an attempt to detect the activity to convert PEP to PnPy was carried out with the purified PEPPM. The assay was performed by using the malate dehydrogenase coupled system described in Materials and Methods. The amounts of PEP converted to PnPy, estimated from the decrease in A_{340} of NADH, were 11.6, 88.4, and 133.6 µM from PEP concentrations of 10, 50, and 100 mM, respectively, showing the purified enzyme to have the activity to form a C-P bond.

Kinetic studies. The kinetic properties of PEPPM were studied with a purified enzyme after the Mono Q step by measuring the initial velocity of the reaction at 25°C. The K_m value of PEPPM for PnPy was calculated to be 19 ± 3.5 µM, and the maximum initial velocity for the conversion of PnPy to PEP was 200 µM/s/mg. Because the equilibrium constant of the C-P bond-forming reaction was too small, the amount of PnPy from PEP was too small to measure the initial velocity of the reaction.

Effects of pH, temperature, and metal ions. The effect of pH on the activity of PEPPM in the presence of Mg^{2+} (5 mM) at 25°C was examined. This enzyme showed a maximum activity in the neutral region between pH 7.5 and 8.0 (Fig. 3A).

The optimum temperature was found to be 40°C, and the enzyme activity was rapidly lost at above 50°C (Fig. 3B).

Activation of PEPPM by metal ions was measured for Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} with an enzyme preparation which had been desalted by gel filtration in the presence of 100 mM PnPy–50 mM Tris-HCl (pH 7.5, 25°C). The apparent K_m values measured for these metal ions (Table 2) revealed that the specificity of the enzyme toward Mn^{2+} was much higher. Suppression of this enhancement by the addition of EDTA indicated that the divalent metal ion is essential for PEPPM activity.
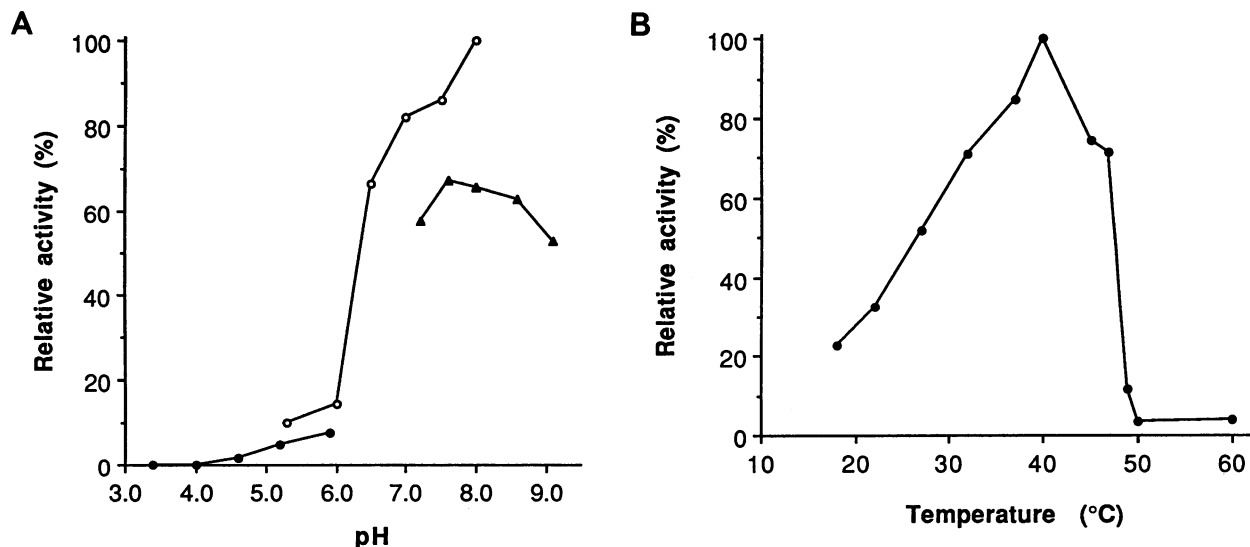
FIG. 3. Effects of pH (A) and temperature (B) on the activity of PEPPM. (A) The reactions were carried out for 15 min at 25°C in the following buffers (final concentration, 50 mM): CH_3COONa-CH_3COOH (●), potassium phosphate (○), and Tris-HCl (▲). (B) The reactions were carried out for 15 min at various temperatures in 50 mM Tris-HCl buffer (pH 7.5).

DISCUSSION

The C-P bond-forming reaction catalyzed by PEPPM is the first step in the biosynthesis of C-P compounds, and this reaction is now widely accepted as common in all of the C-P compound-producing organisms. So far, this enzyme has been purified from only two organisms, T. pyriformis and S. hygroscopicus. In order to reveal the nature of this unique enzyme in more detail, we purified PEPPM from a gram-negative bacterium, P. gladioli B-1, for the first time. The PEPPM from P. gladioli is a homotetramer of 61-kDa proteins whose size is considerably different from those of PEPPM from T. pyriformis (a homodimer with a subunit molecular mass of 32 kDa) and S. hygroscopicus (with a subunit molecular mass of 33.7 kDa deduced from the nucleotide sequence of the corresponding gene) (4). There is significant homology between the amino acid sequences of PEPPM from the gram-positive bacterium S. hygroscopicus and PEPPM from the eukaryote T. pyriformis (21), although they are not close relatives. Nevertheless, the molecular size of the PEPPM protein of P. gladioli, which is evolutionarily more closely related to Streptomyces species than to Tetrahymena species, is surprisingly very different from those of PEPPM from Streptomyces and Tetrahymena species. Cloning and sequencing of the PEPPM gene of P. gladioli are now in progress and will be reported elsewhere, along with the amino acid sequence of the PEPPM protein. The analysis of the PEPPM gene of P. gladioli will reveal whether the homologous sequence is included as a universal sequence of

the PEPPM gene and will help the analysis of the active site of the enzyme.

PEPPM from P. gladioli showed the same characteristics as those from S. hygroscopicus (8) and T. pyriformis (2, 20). It needs the divalent metal ion for activity, and the equilibrium of the catalyzed reaction favors cleavage of the C-P bond of PnPy to PEP. The K_m value for PnPy of PEPPM from P. gladioli was the same as that for PnPy of PEPPM from T. pyriformis (2, 20, 21). The C-P bond-forming activity of this enzyme was confirmed by measuring PnPy production with malate dehydrogenase-NADH in the same way as PEPPM of S. hygroscopicus was measured previously (8). Although PEPPM activity favors the formation of PEP from PnPy, organisms can produce C-P compounds efficiently. Thus, there should be a mechanism to promote the conversion of PEP to PnPy. The second step of the reaction is assumed to be decarboxylation of PnPy to phosphonoacetaldehyde to irreversibly drive the reaction forward (Fig. 1). In order to elucidate the formation mechanisms of C-P compounds in living systems, it is necessary to make clear the unknown mechanism of the next step of the reaction following C-P bond formation, which is now in progress.

PEPPM from P. gladioli was very unstable under acidic conditions, and under the culture conditions reported here, the pH of the medium was very low, remaining at around pH 3 throughout the steady-state phase. When another medium in which the pH was constantly neutral was used, the production of the C-P compound hydroxylethylphosphonic acid did not increase and the activity of PEPPM reached a high level only at the early log phase and was rather weak. PEPPM stability was very sensitive to temperature, and even at 35°C, about half of the enzyme activity was lost in 30 min. This is in agreement with a previous finding of the optimum culture temperature (27°C) for the production of hydroxylethylphosphonic acid (17). In metal ion activation experiments, Zn^{2+} seemed to be the most effective in activating PEPPM, but the addition of Zn^{2+} to the growth medium did not have any effect on the production of the C-P compound.

TABLE 2. Kinetic constants for metal ion activation

Metal ion	$V_{max}/V_{max}(Mg^{2+})^a$	K_m (μM)
Mg^{2+}	1.0	3.5 ± 1.4
Mn^{2+}	0.5	$(1.6 \pm 0.5)10^{-2}$
Zn^{2+}	2.3	3.0 ± 1.5
Co^{2+}	0.9	1.2 ± 0.2

^{a} $V_{max}/V_{max}(Mg^{2+})$, ratio of V_{max} for each metal ion under the conditions described in the text to that for Mg^{2+}.

Pseudomonas syringae (22) and *Pseudomonas viridiflava* (15) were identified as C-P compound producers, which respectively produced fosfomycin and fosfadecin, which has a hybrid structure consisting of fosfomycin and AMP. Fosfomycin is also produced by *Streptomyces wedmorensis* (5) and *Streptomyces fradiae* (19). There is considerable interest in comparing the PEPPM protein of these fosfomycin-producing *Pseudomonas* species and those of *P. gladioli* and *Streptomyces* species.

Although the C-P bond-forming activity of *S. hygroscopicus* is high, its PEPPM is too unstable to be completely purified, and other C-P compound-producing *Streptomyces* species showed very weak enzymatic activities. Therefore, *P. gladioli* and its PEPPM, presented here, are expected to be useful for protein structural studies and analysis of the step following the PEPPM reaction.

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