STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293)

8. PURIFICATION AND CHARACTERIZATION OF 2-PHOSPHINOMETHYLMALIC ACID SYNTHASE FROM *STREPTOMYCES HYGROSCOPICUS* SF-1293†

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2-Phosphinomethylmalic acid (PMM) synthase catalyzes the condensation of phosphinopyruvic acid (PPA), an analog of oxalacetic acid, and acetyl-CoA to form PMM. The enzyme was purified approximately 700-fold from a cell-free extract of *Streptomyces hygroscopicus* SF-1293, a bialaphos producing organism, to an electrophoretically homogeneous state. The purified PMM synthase has a subunit molecular weight of 48,000 by SDS-polyacrylamide gel electrophoresis and a native molecular weight of 90,000~98,000 by gel filtration. PMM synthase was relatively unstable, showed maximum activity at pH 8.0 and 30°C, and was inhibited strongly by *p*-chloromercuribenzoate, iodoacetamide and EDTA. Enzyme activity suppressed by EDTA was completely restored by adding Co^{++} or Mn^{++} and partially restored by addition of Ca^{++}, Fe^{++} or Mg^{++}.

The specific substrates of this enzyme are PPA or oxalacetic acid in addition to acetyl-CoA. The enzyme does not catalyze the liberation of CoA from acetyl-CoA in the presence of α-keto acids, such as pyruvate, α-ketoglutarate, deamino-α-ketodemethylphosphinothricin or phosphonopyruvate. The condensation reaction did not take place when propionyl-CoA or butyryl-CoA was used as a substrate in place of acetyl-CoA. The *Km* values of the enzyme were 0.05 mM for acetyl-CoA, 0.39 mM for PPA and 0.13 mM for oxalacetate. PMM synthase is very similar to (*R*)-citrate synthase of *Clostridium* in the inhibition pattern by sulfhydryl compounds, its metal ion requirement and stereospecificity; unlike (*R*)-citrate synthase PMM synthase was not inhibited by oxygen.

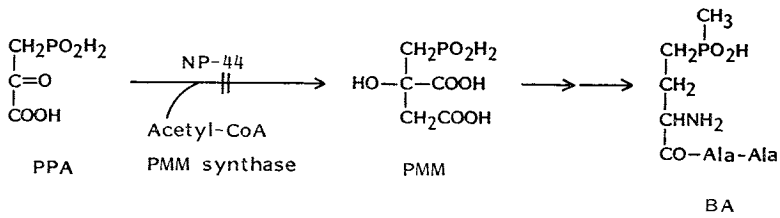
Bialaphos (BA) is a metabolite produced by *Streptomyces hygroscopicus* SF-1293[2,3] and is now in use as a herbicide. The very unusual C-P-C bond in its structure††,[5] has prompted us to study the mechanism of its biosynthesis. We have reported the biosynthetic pathway of BA which comprises at least 13 steps based on several experiments using ^{13}C-labeled precursors[6], blocked mutants[7,8] and metabolic inhibitors[9].

During these studies, 2-phosphinomethylmalic acid (PMM) was isolated from the fermentation broth supplemented with mono-fluoroacetic acid[9]. Subsequent experiments proved that PMM is

† For part 7[1].

†† For reviews of C-P compounds[4].

Fig. 1. Conversion of phosphinopyruvic acid (PPA) to bialaphos (BA) *via* 2-phosphinomethylmalic acid (PMM).



a biosynthetic intermediate of BA formed by the condensation of phosphinopyruvic acid (PPA) and acetyl-CoA (Fig. 1). In view of the structural similarity of PMM and PPA with citric acid and oxalacetic acid, respectively, this condensation reaction was thought to be comparable with that catalyzed by citrate synthase. In fact, the absolute stereochemistry of PMM was determined to be (*R*)† by comparison with (*S*)-PMM prepared from PPA and acetyl-CoA by the action of (*S*)-citrate synthase of porcine heart origin[1]. These results strongly suggested that PMM synthase may be closely related to (*R*)-citrate synthase isolated from a few obligate anaerobic bacteria[10~12].

In this paper, the isolation of PMM synthase and comparison of its properties with citrate synthases are described in detail.

Materials and Methods

Microorganism

S. hygroscopicus SF-1293 was used throughout this work. The microorganism was cultivated at 27°C for 72 hours on a rotary shaker in a 500-ml flask containing 100 ml of a medium with the following composition; glucose 7%, Bacto-soytone 4.4%, KH_2PO_4 0.327%, Na_2HPO_4 0.085%, TES 1.15% and $CoCl_2 \cdot 6H_2O$ 0.0001%, pH 6.0. The blocked mutant NP-44, which could not produce PMM, was used to assay the transformation of [^{14}C]PMM to BA. It was cultivated at 27°C in P-2 medium consisting of glycerol 3.0%, yeast extract 3%, KH_2PO_4 0.1%, $CoCl_2 \cdot 6H_2O$ 0.0001%, pH 7.0. Four days after inoculation, the mycelium was collected by centrifugation (15,000×*g*, 10 minutes).

Enzyme Assay

Two procedures were employed to assay PMM synthase activity and PMM transforming activity.

Radioactive Assay: (a) Assay of PMM synthase; The reaction mixture contained the following in a final volume of 24 μl: 20 μl of crude PMM synthase of *S. hygroscopicus* SF-1293 (20~40% ammonium sulfate precipitate) in 50 mM Tris-HCl buffer (pH 7.5), 2 μl of 0.1 mM PPA and 2 μl of 1 mM [1-^{14}C]acetyl-CoA (0.24 mCi/mmol). The mixture was incubated at 30°C for 2 hours, boiled in a water bath for 3 minutes to stop the reaction and then analyzed by TLC on cellulose plates. The reaction products were detected autoradiographically.

(b) Assay of BA synthesis; Two kinds of ^{14}C-labeled PMMs, one formed by the above method and the other from PPA and [1-^{14}C]acetyl-CoA by citrate synthase of porcine heart origin, were separately incubated with cells of NP-44 at 30°C for 20 hours. The reaction mixtures were analyzed in the same way as for the assay (a).

Spectrophotometric Assay: The assay mixture contained the following in a final volume of 1 ml; 0.1 M Tris-HCl buffer (pH 8.0), 0.1 mM 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB), 0.2 mM acetyl-CoA, 0.25 mM PPA and crude enzyme, and was incubated at 30°C. The reaction was followed by the increase in absorbance at 412 nm.

† In the previous paper[1], we reported the absolute stereochemistry of PMM to be (*S*) due to an error in nomenclature. Since the ORD spectrum of synthetic PMM prepared by the action of (*S*)-citrate synthase is opposite to that of natural PMM, the stereochemistry of synthetic PMM should also be amended to (*S*).

The spectrometric method and radioactive assay were used in parallel to confirm the formation of PMM at the first step of the purification procedure. One U of the enzyme catalyzes the formation of 1 μmol of CoA per minute at 30°C. Kinetic measurements of the PMM synthase reaction were performed using the spectrophotometric assay. Amounts of protein were determined by the method of LOWRY *et al.*

Identification of Reaction Products

The reaction mixtures in the radioactive assay were applied to cellulose thin layer plates (10×10 cm, Merck) and developed with BuOH - CH_3COOH - H_2O (2 : 1 : 1) in the first dimension and C_2H_5OH - NH_4OH - H_2O (8 : 1 : 4) in the second dimension. In the first dimension, Rf values were 0.35 (PPA), 0.36 (acetyl-CoA), 0.43 (PMM) and 0.58 (BA). PMM, deamino-α-ketodemethylphosphinothricin (DKDPT), *N*-acetylphosphinothricin, PPA and citrate were detected by the $FeCl_3$-sulfosalicylic acid color reaction, while glutamic acid, phosphinothricin and BA were detected with ninhydrin color reaction.

Purification of PMM Synthase

All purification procedures were carried out at 0~4°C.

Step 1. Sonic Treatment: Mycelia of the parent strain were harvested by centrifugation at 15,000×*g* for 10 minutes. The cells were washed twice with Tris-buffer (50 mM Tris-HCl, pH 7.5) by centrifugation. Finally, 184 g of the washed cells were suspended in 910 ml of Tris-buffer and sonicated with a 600-W ultrasonic disintegrator (Toyo Rika Co., 2N-100) for 8 minutes. Cell debris were removed by centrifugation at 15,000×*g* for 30 minutes.

Step 2. Ammonium Sulfate Precipitation: Solid ammonium sulfate was added to the cell-free extract prepared in Step 1 and the fraction precipitating between 20~40% saturation was collected. About 48% of the enzyme activity in the cell-free extract was recovered in this fraction. The precipitate was dissolved in Tris-buffer, and dialyzed against the buffer for 20 hours.

Step 3. DEAE-cellulose Fractionation: The dialyzed ammonium sulfate fraction was applied to a DE-52 cellulose column (2.8×45 cm), equilibrated with Tris-buffer. The column was washed with 220 ml of the buffer and the enzyme was then eluted with Tris-buffer (2 liters) supplemented with KCl in a linear concentration gradient from 0 to 1 M at a flow rate of 110 ml/hour. The active fraction was precipitated by adding solid ammonium sulfate to give a 50%-saturated solution. After centrifugation at 15,000×*g* for 30 minutes, the precipitate was dissolved in 25 ml of 50 mM Tris-HCl buffer and dialyzed against the same buffer for 20 hours.

Step 4. Gel Filtration: The solution from Step 3 was loaded on a column (3.0×64 cm) of Sephadex G-200 which had been equilibrated with Tris-buffer. The column was eluted in 5 ml fractions at a flow rate of 6 ml/hour. Fractions containing PMM synthase activity were combined and the enzyme was precipitated with 50%-saturated ammonium sulfate. The pellet was collected by centrifugation, dissolved in Tris-buffer and dialyzed against 20 mM Tris-HCl buffer, pH 7.5 for 20 hours.

Step 5. Affinity Chromatography using Matrex Gel Red A: The enzyme fraction obtained in Step 4 was applied to a Matrex Gel Red A column (1.4×16 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, and the column was allowed to stand for 30 minutes. After washing with the same buffer (75 ml), the column was eluted with the buffer containing KCl in a linear concentration gradient from 0 to 2 M (200 ml) at a flow rate of 14 ml/hour. The enzyme fractions were combined, precipitated with solid ammonium sulfate to give a 50%-solution and dialyzed as described in Step 2.

Step 6. The Second Gel Filtration: The enzyme preparation obtained in Step 5 was purified by gel filtration using a Sephadex G-100 column (1.7×91 cm) as in Step 4. Fractions containing PMM synthase activity were combined, concentrated by methods ultrafiltration and lyophilized. The powder obtained was kept at 4°C.

Determination of the Molecular Weight by Gel Filtration

Gel filtration was carried out with Sephadex G-100 (fine, Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The standard proteins used were ovalbumin, bovine serum albumin and

γ-globulin with molecular weights of 45,000, 67,000 and 160,000, respectively.

Chemicals

Tris and DTNB were purchased from Wako Pure Chemical Industries, Ltd.; acetyl-CoA and porcine heart citrate synthase were obtained from Sigma Chemical Co. The three standard protein markers used for gel filtration were purchased from Schwarz/Mann Co. and the protein marker mixtures used for SDS-polyacrylamide gel electrophoresis was purchased from Pharmacia Fine Chemicals. [1-^{14}C]Acetyl-CoA of specific activity 0.24 mCi/mmol was supplied from Amersham International.

Results

Purification of PMM Synthase

The formation of PMM was catalyzed by crude cell-free extracts of *S. hygroscopicus* SF-1293 in the presence of PPA and acetyl-CoA. PMM synthase was purified from the cells of *S. hygroscopicus* SF-1293 as described in Materials and Methods. In Step 2, PMM synthase was separated from citrate synthase which remained in the supernatant fraction. A single peak with PMM synthase activity was obtained by elution with 0.7 to 1 M KCl from Matrex Gel Red A column in Step 5 (data not shown) and was followed by purification on a Sephadex G-100 column in Step 6 (Fig. 2). With crude extracts or less purified preparations, the enzyme activity decreased rapidly during freezing and thawing. The enzyme fraction stored as a lyophilized powder at 4°C lost 5% of its activity within 3 months. For measurement of the enzyme activity at each purification step, the lyophilized stored preparation was used. The procedures for a typical purification are summarized in Table 1. The enzyme was purified approximately 700-fold with an overall yield of 4.3% from a cell-free extract.

Identification of Reaction Product

The radioactive reaction product formed when partially purified PMM synthase was incubated with PPA and [1-^{14}C]acetyl-CoA was identical in Rf value by cellulose TLC with authentic PMM.

Fig. 2. Elution profile of 2-phosphinomethylmalic acid (PMM) synthase from a Sephadex G-100 column.
— A_{280}, ● PMM synthase activity (A_{412}).

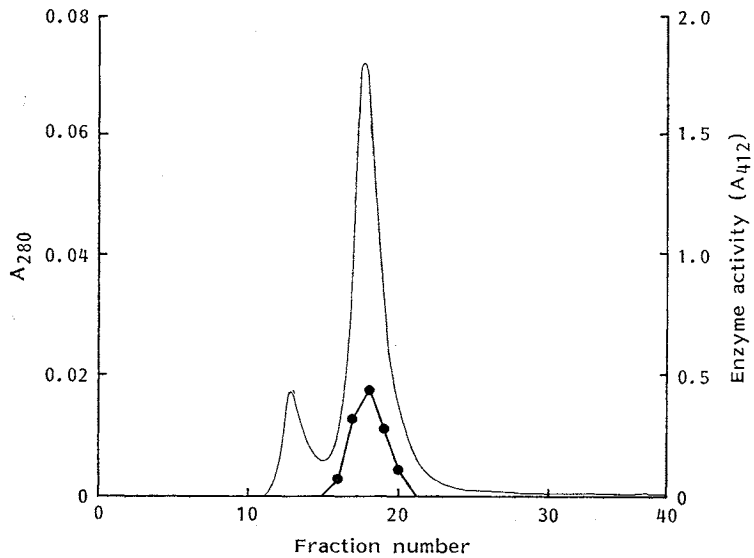
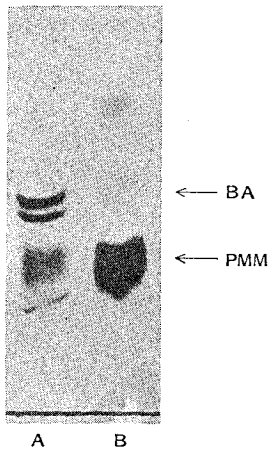


Table 1. Purification of PMM synthase.

Step	Total protein (mg)	Total U	Specific activity (U/mg protein)	Yield (%)
1. Crude extract	7,462	5,926	0.79	100
2. Ammonium sulfate	2,098	2,838	1.35	48
3. DEAE-cellulose	326	1,765	5.4	30
4. Sephadex G-200	54.4	743	13.7	12.5
5. Matrex Gel Red A	1.05	471	448.6	7.9
6. Sephadex G-100	0.47	257	546.8	4.3

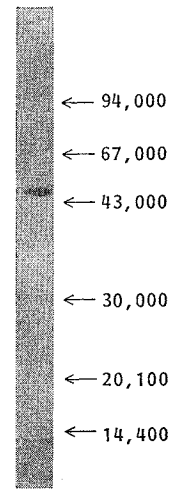
1 U=1 μmol substrate/minute.
Details of the procedures are given in Materials and Methods.

Fig. 3. Cellulose TLC autoradiograph of the reaction products formed by the washed mycelia of NP-44 from ^{14}C-labeled 2-phosphinomethylmalic acids (PMMs).



PMMs synthesized by crude PMM synthase (A) or porcine heart citrate synthase (B).
BA: Bialaphos.

Fig. 4. SDS-PAGE of purified 2-phosphinomethylmalic acid (PMM) synthase.



Purified PMM synthase from Sephadex G-100 was subjected to SDS-PAGE (10~20% gradient of acrylamide). The gel was stained with Coomassie Brilliant Blue R-250. The amount of the enzyme used was 9 μg. The arrows indicate the marker dyes.

Since (*S*)-citrate synthase of porcine heart catalyzed the synthesis of PMM at a very poor rate, the configuration of PMM synthesized by PMM synthase was analyzed by the following method. The [^{14}C]PMM was incubated with the blocked mutant NP-44 which could not produce PMM and therefore could not produce BA. A radioactive spot was observed at the same position as BA (Fig. 3). However, (*S*)-PMM prepared with (*S*)-citrate synthase was not utilized as the substrate to form BA in this system. Thus, it is confirmed that the configuration of PMM synthesized by PMM synthase differs from that of (*S*)-PMM.

General Properties of the Enzyme

Analysis of the final enzyme preparation by SDS-PAGE gave one predominant protein band (Fig. 4), and HPLC analysis showed only one peak (data not shown). Thus, the enzyme prepared in Steps 1 to 6 is believed to be homogeneous. PMM synthase activity was measured at various pH values using 25 mM sodium phosphate buffer (pH 5.5~7.5) and 0.1 M Tris-HCl buffer (pH 7.5~10.0).

The optimal pH for enzyme activity was 8.0 (Fig. 5). Although enzyme activity was not affected by 10 mM phosphate buffer, it was suppressed by 50% in 100 mM phosphate buffer.

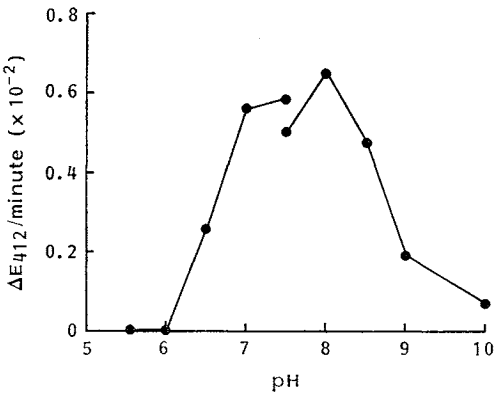
Estimation of the Molecular Weight

The molecular weight of the enzyme by gel filtration was estimated to be 90,000~98,000 (Fig. 6). The final preparations of PMM synthase gave a single protein band by SDS-PAGE. Comparison of its Rf value with those of molecular weight standards on a 10~20%-gradient SDS-PAGE demonstrated the subunit molecular weight to be 48,000 (Fig. 4). Therefore, the protein is believed to be dimeric.

Substrate Specificity

PMM synthase showed no activity when acetyl-CoA was substituted by propionyl-CoA or butyryl-CoA. α-Keto acid derivatives such as pyruvate, phosphonopyruvate, α-keto glutarate and DKDPT could not replace PPA in the PMM synthase reaction. However, oxalacetic acid was a good substrate for PMM synthase and was utilized approximately 70 times as efficiently. This experimental result shows that in addition to PPA, PMM synthase itself has ability to utilize

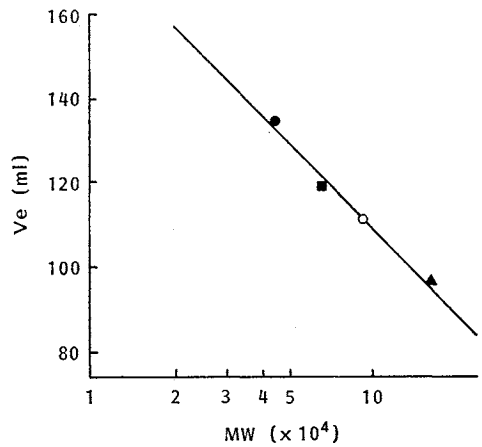
Fig. 5. Effects of pH on 2-phosphinomethylmalic acid synthase activity.



Sodium phosphate buffer was used between pH 5.5 and 7.5, and Tris-HCl buffer was used between pH 7.5 and 10.0.

Fig. 6. Determination of the molecular weight by Sephadex G-100 gel filtration.

● Ovalbumin, ■ bovine serum albumin, ○ 2-phosphinomethylmalic acid (PMM) synthase, ▲ γ-globulin.



PMM synthase (3 mg) and marker proteins (2 mg each) were applied to a column of Sephadex G-100 (1.7×91 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.5. The column was eluted with the same buffer, the optical density of the eluate being monitored in 5 ml fractions; the enzyme activity in each fraction was measured as CoA released from acetyl-CoA and Phosphinopyruvic acid, determined at 412 nm.

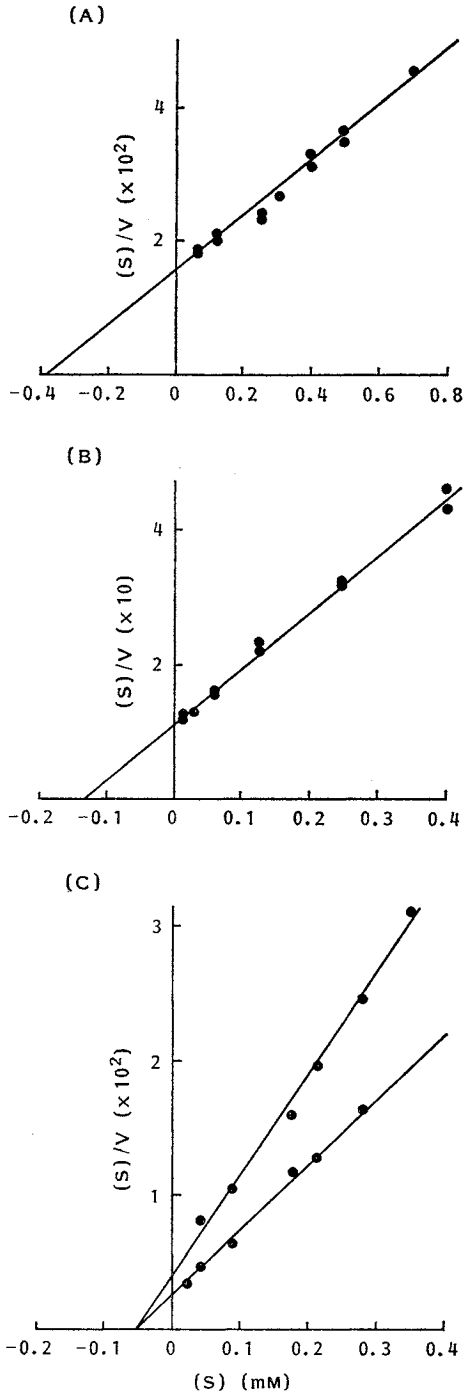
Table 2. Effect of *p*-chloromercuribenzoate (pCMB), iodoacetamide (IA) and EDTA on the activity of 2-phosphinomethylmalic acid (PMM) synthase and citrate synthases.

Enzyme	Activity (%) with pCMB (0.1 mM)	Activity (%) with IA (1 mM)	Activity (%) with EDTA (0.05 mM)
PMM synthase	2	18	0
(*R*)-Citrate synthase[b]	12	10[a]	0
(*S*)-Citrate synthase[b]	95	84[a]	100

[a] 0.1 mM IA.

[b] Data taken from ref 15.

Fig. 7. Estimation of *Km* values of phosphinopyruvic acid (PPA) (A), oxalacetic acid (B), and acetyl-CoA (C) by Hanes-Woolf plot.



The initial rate of 2-phosphinomethylmalic acid synthase activity was measured using various concentrations of PPA, oxalacetic acid or acetyl-CoA.

Table 3. Effect of metal ions on PMM synthase, (*R*) and (*S*)-citrate synthase activity.

Metal ion added (1 mM)	PMM synthase activity (%)	(*R*)-Citrate[15] synthase activity (%)	(*S*)-Citrate[15] synthase activity (%)
None	100	100	100
EDTA	15	0	100
Mn^{++}	100	100	
Co^{++}	100	100	
Mg^{++}	44	14	
Ca^{++}	54	25	
Fe^{++}	50	25	
Zn^{++}	24	16	
Zn^{++}[a]	46	45	

[a] 0.01 mM.

oxalacetate as a substrate.

Inhibition by *p*-Chloromercuribenzoate (pCMB) and Iodoacetamide (IA)

The effects of pCMB and IA on the activity of PMM synthase are shown in Table 2. At a concentration of 0.1 mM of pCMB or 1 mM of IA, the enzyme was inhibited by 98 and 82%, respectively. (*R*)-Citrate synthase from *Clostridium acidiurici*[13,14] was almost completely inhibited by pCMB at a concentration of 50 μM. (*S*)-Citrate synthase from pig heart, on the other hand, was resistant to those reagents[14]. According to EGGERER and REMBERGER[15], pCMB up to a concentration of 2 mM did not inhibit (*S*)-citrate synthase activity.

Km Values for Acetyl-CoA, PPA and Oxalacetate

The *Km* values were determined to be 52 μM for acetyl-CoA, 385 μM for PPA and 130 μM for oxalacetate (Fig. 7).

Metal Ion Requirement

Inhibition of PMM synthase by EDTA is shown in Table 2. The enzyme was inhibited almost completely by 50 μM of EDTA. The 85% inhibition of PMM synthase by 25 μM EDTA was completely relieved by addition of Mn^{++} or Co^{++} ions and partially relieved by Mg^{++},

Ca^{++} or Fe^{++} ions at a concentration of 1 mM. Low concentrations of Zn^{++} also activated the enzyme but higher concentrations were inhibitory (Table 3). A similar tendency for metal ion requirements was reported for (*R*)-citrate synthase[14,16].

Discussion

PMM synthase, the enzyme involved in the condensation of PPA and acetyl-CoA, has been purified by approximately 700-fold and several of its biochemical properties have been investigated. The product of this enzyme reaction is PMM in which one of the carboxylic acids of citric acid is replaced by a phosphinic acid function. The stereochemical course of the reaction is opposite to that of the ubiquitous (*S*)-citrate synthase[1], and therefore, PMM synthase may be regarded as belonging to the group of (*R*)-citrate synthases like that isolated from *C. acidiurici* by GOTTSCHALK and DITTBRENNER[14]. In fact, PMM synthase showed very close similarity to (*R*)-citrate synthase with regard to metal ion requirement and sensitivity to enzyme inhibitors such as EDTA[14,16], pCMB[13,14] and IA[14]. Inhibition of PMM synthase by EDTA was completely restored by Mn^{++} or Co^{++}, and partially by Ca^{++}, Fe^{++} or Mg^{++}. On the other hand, (*S*)-citrate synthases from sources such as animal tissues, yeast and *Escherichia coli* are not inhibited by EDTA, and thus the metal ions are not necessary to its activity.

It is interesting to postulate that PMM synthase and (*R*)-citrate synthase may be identical in evolutionary origin; however unlike the latter, the former enzyme is not inactivated by molecular oxygen. It is, therefore, important to compare the amino acid sequence of these two enzymes. Comparison of the NH_2-terminal amino acid sequence between PMM synthase and citrate synthase of *S. hygroscopicus* SF-1293 has revealed no similarity between them. This along with the purification of citrate synthase of *S. hygroscopicus* SF-1293 will be reported elsewhere.

Acknowledgments

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