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PARTIAL PURIFICATION AND PROPERTIES OF PHOSPHOENOLPYRUVATE CARBOXYKIN ASE OF *ESCHERICHIA COLI* W

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SUMMARY

Phosphoenolpyruvate carboxykinase was purified 85-fold in a yield of 24% over cell-free extract of *Escherichia coli* W. The molecular weight was estimated to be 64,000 by the method of gel filtration.

When assayed in a direction of CO_2-fixation, the enzyme showed a pH optimum at 7.0 and required Mn^{2+} for the activity (Km=0.6 mM). Km values for phosphoenolpyruvate, ADP and $KHCO_3$ were 0.6, 0.4 and 40 mM, respectively.

When assayed in a direction of decarboxylation, Km value for oxaloacetate was 0.6 mM. The plots of reaction velocity against ATP concentration showed a characteristic curve for substrate inhibition at higher concentrations than 1 mM. Km value for ATP was about 0.2 mM.

The enzyme activity was not affected by the metabolites tested, including intermediates of the glycolytic pathway and the tricarboxylic acid cycle, and pyridine nucleotides.

Introduction

Phosphoenolpyruvate carboxykinase [EC 4.1.1.32] which catalyzes PEP**-formation reaction from oxaloacetate and ATP is known to be a key enzyme in gluconeogenesis (1). Recent studies on the levels of the enzyme of *Escherichia coli* grown under various nutritional conditions by Hsie and Rickenberg (2), Shrago and Shug (3), and by us (4) indicate that the level of the enzyme is high in gluconeogenesis and low in glycolysis. Wright and Sanwal (5), using two assay methods, *i.e.* isotope exchange assay and CO_2-fixation assay, reported that the partially purified enzyme from *E. coli* B is inhibited by NADH in an allosteric manner.

In this communication, a partial purification of PEP carboxykinase from *E. coli* W and kinetic properties of the enzyme in the reactions of both decarboxylation

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** Abbreviations: PEP, phosphoenolpyruvate; DTT, dithiothreitol; Tris, tris (hydroxylmethyl)-aminomethane.

and CO_2-fixation directions are described. Regulatory mechanism of the enzyme is also discussed.

MATERIALS AND METHODS

Chemicals

Malate dehydrogenase [EC 1.1.1.37] was obtained from Boehringer-Mannheim. DTT, Tris, bovine serum albumin, NADH, NAD^+ and Fast violet B were Sigma products. Sephadex G-100 and DEAE-Sepahdex were obtained from Pharmacia. PEP was prepared by the method described previously (6). All other chemicals were purchased from Nakarai Chemicals Co. (Kyoto).

Preparation of Cell-free Extract

E. coli W was grown aerobically with shaking at 30°C in a medium containing 10 g of polypeptone, 10 g of yeast extract and 5 g of K_2HPO_4 in 1 l of distilled water. The cells were harvested near the end of the logarithmic growth phase and washed twice with 1% KCl solution. The cells suspended in 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.1 mM DTT (0.5 g wet cells/ml) were disrupted with a Ohtake sonic disintegrator at 20 kHz for 10 min at 4°C. This suspension was centrifuged at 25,000×g for 40 min and the resulting supernatant was used as the enzyme source for purification.

Enzyme Assays

1. Assay of the enzyme in the direction of CO_2-fixation—The standard reaction mixture contained 5 μmoles of PEP, 5 μmoles of ADP, 10 μmoles of $MnSO_4$, 50 μmoles of $KHCO_3$, 0.15 μmole of NADH, 1 I.U. of malate dehydrogenase, 0.2 μmole of DTT and 70 μmoles of Tris-maleate buffer, pH 7.0, in a total volume of 1.0 ml. The enzyme activity was determined at 30°C by following the rate of NADH oxidation at 340 nm in a Hitachi 124 spectrophotometer, as in the case of PEP carboxylase (7).

2. Assay of the enzyme in the direction of decarboxylation—The standard reaction mixture contained 5 μmoles of oxaloacetate, 2 μmoles of ATP, 80 μmoles of $MgSO_4$, 0.2 μmole of DTT and 70 μmoles of Tris-maleate buffer, pH 7.0, in a total volume of 1.0 ml. The mixture was incubated at 30°C for 10 min and then it was cooled in an ice bath to stop the enzyme reaction. The enzyme activity was assayed by determination of inorganic phosphate which was liberated from PEP, one of the reaction products, by the addition of 0.1 ml of 5% $HgCl_2$ to the reaction mixture (8). Inorganic phosphate was determined by the method of Yoda (9).

One unit of the enzyme was defined as the amount of producing 1 μmole of product per min under the assay conditions.

Protein Determination

Protein concentration was determined by the method of Lowry *et al.* (10) with crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of the Enzyme

All operations were performed at 0–4°C. The crude cell-free extract was diluted with 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.4 mM DTT to a final concentration of 10 mg protein per ml and the solution was treated with 0.25 volume of 1% protamine sulfate solution. The suspension was stirred for 10 min and centrifuged at 15,000×g for 10 min. To the supernatant solution was added solid ammonium sulfate and the fraction precipitating between 55 to 75% saturation was dissolved in a minimal volume of 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.4 mM DTT. The enzyme solution was dialyzed overnight against 170 volumes of 0.01 M potassium phosphate buffer, pH 6.0, containing 0.1 mM DTT. Suspension of calcium phosphate gel washed twice with 0.05 M potassium phosphate buffer, pH 6.0, containing 0.1 mM DTT (20 mg dry weight/ml) was added to the dialyzate in a proportion of 2 mg dry gel to 1 mg protein with gentle stirring. The mixture was further stirred for 5 min and then centrigufed to pack the gel. After the gel was washed once with 0.05 M potassium phosphate buffer, pH 6.0, containing 0.1 mM DTT, the combined supernatant was brought to 80% saturation with solid ammonium sulfate. The precipitated fraction was dissolved in 0.05 M Tris-HCl buffer, pH 7.7, containing 0.05 M KCl, 1 mM EDTA and 0.1 mM DTT. The enzyme solution was dialyzed overnight against 180 volumes of the same buffer and applied to a DEAE-Sephadex column (1.5×40 cm) previously equilibrated with the buffer. The enzyme was eluted with a linear gradient of KCl concentration from 0.05 to 0.5 M in 0.05 M Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 0.4 mM DTT and fractions of 4 ml were collected (Fig. 1). The fractions from 32 to 39 were pooled and solid ammonium sulfate was added to the solution to a level of 80% saturation. The precipitate was centrifuged and dissolved in a minimal volume of 0.1 M Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 0.4 mM DTT. The enzyme solution was applied to a Sephadex G-100 column (1.1×60 cm) previously equilibrated with the same buffer. The enzyme was eluted with the buffer and 0.6-ml fractions were collected. Fractions with the

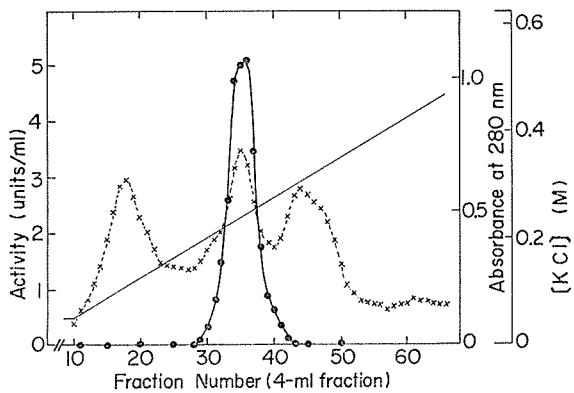

Fig. 1. Elution pattern of PEP carboxykinase from DEAE-Sephadex column. —●— Enzyme activity; —×—, absorbance at 280 nm; ———, KCl concentration.

specific activity higher than 8 units per mg of protein were pooled and used for the experiments described below.

Table 1 shows one of the typical results of the purification. PEP carboxykinase was purified 85-fold over the crude cell-free extract of *E. coli* W.

Table 1. Purification of PEP carboxykinase

Step	Protein (mg)	Activity[a] (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1. Crude extract	3058	323	0.11	100	1
2. Protamine sulfate	1826	319	0.17	99	1.5
3. $(NH_4)_2SO_4$ (55–75% saturation)	520	276	0.43	86	3.9
4. Calcium phosphate gel	176	189	1.1	59	10
5. DEAE-Sephadex chromatography	15	115	7.7	36	70
6. Sepahdex G-100 chromatography	8.4	78.0	9.3	24	85

a) Assayed in a direction of CO_2-fixation reaction.

Kinetic Properties

1. CO_2-fixation reaction—pH optimum of CO_2-fixation reaction was 7.0 in 0.07 M Tris-maleate buffer. The activity was about one-half its maximum value at pH 5.5 and about one-fifth at pH 8.0. As shown in Fig. 2A, Mn^{2+} was essential for the enzyme activity and the Km value was 0.6 mM. When Mg^{2+} was used instead of Mn^{2+}, the enzyme activity attainable at its infinite concentration was only 10–20% of that attainable with Mn^{2+}. Figure 2 (B-D) shows effects of varying concentrations of substrates on the reaction velocity. The curve showing the reaction velocity *vs.* PEP concentration was hyperbolic and the Km value for PEP was 0.6 mM (Fig. 2B). As for ADP (Fig. 2C) and $KHCO_3$ (Fig. 2D), curves showing the reaction velocity *vs.* the substrate concentrations were hyperbolic and Km values or ADP and $KHCO_3$ were 0.4 mM and 40 mM, respectively.

It is interesting to consider the relation between the physiological function of the enzyme and its kinetic properties. *In vivo* system of *E. coli*, the enzyme reaction is presumed not to operate in a direction of CO_2-fixation, because a mutant devoid of PEP carboxylase [EC 4.1.1.31] which catalyzes the reaction for replenishing oxaloacetate from PEP has been reported to be unable to grow on glucose, glycerol or pyruvate as sole carbon source in spite of the possession of PEP carboxykinase (11). This fact suggests that a certain unknown mechanism is involved in the control of PEP carboxykinase reaction in the cells (4). When considered the result obtained above, it seems possible that the low affinity of PEP carboxykinase for $KHCO_3$ contributes significantly to the control of the enzyme reaction in the CO_2-fixation direction *in vivo*. In the *E. coli* B enzyme, Km values for ADP and PEP were reported to be 0.23 mM and 14 mM, respectively (5).

2. Decarboxylation reaction—In the experiments for the decarboxylation reaction, Mg^{2+} was used as bivalent cation, because Mn^{2+} accelerated the non-enzymatical decarboxylation of oxaloacetate. The pH profile of the reaction velocity was considerably broad in a range of 6.0 to 8.0, showing a pH optimum of 7.0. Figure 3A shows oxaloacetate-saturation curve of the reaction velocity, indicating

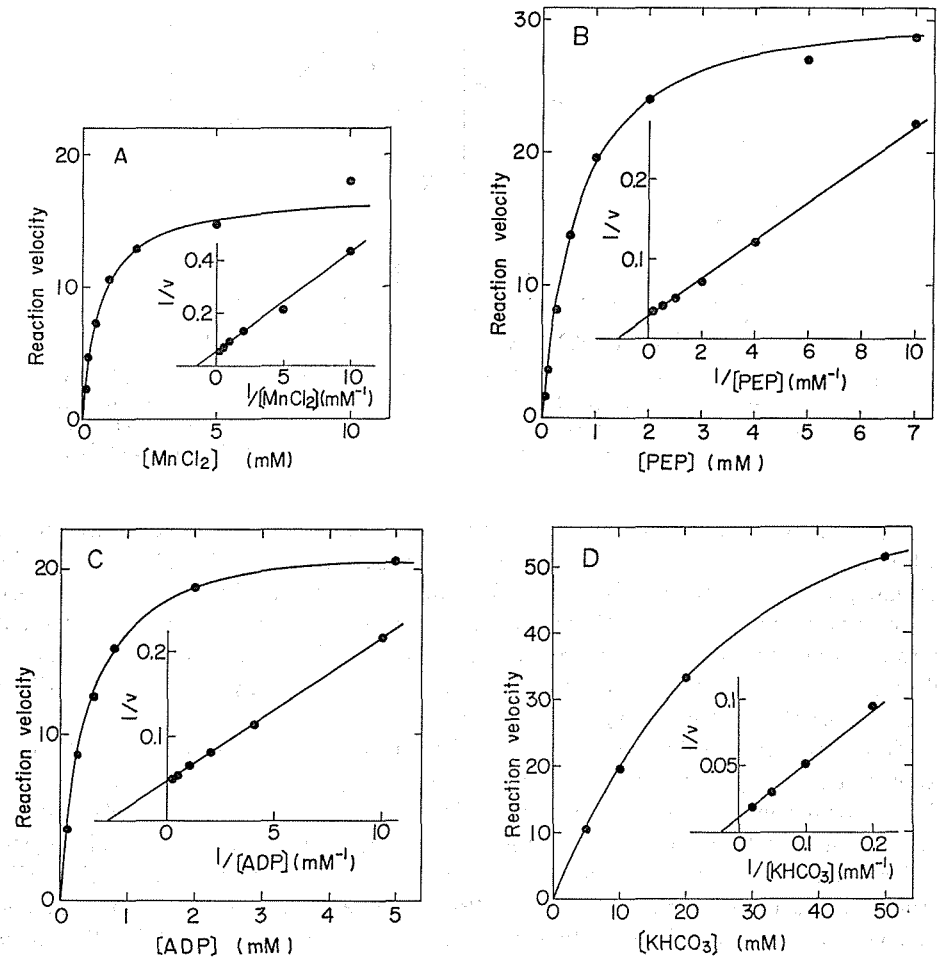

Fig. 2. Effects of $MnCl_2$, PEP, ADP and $KHCO_3$ on the CO_2-fixation reaction of PEP carboxykinase. The reaction mixtures in A, B, C and D contained 2, 3, 2 and 5 μg protein of the enzyme, respectively. Other experimental conditions were as described in "Materials and Methods" except for the use of ligand concentrations as indicated in the abscissa. Reaction velocity was expressed as nmoles of CO_2-fixed per min. Insets represent double reciprocal plots of reaction velocity and ligand concentration.

Km value for oxaloacetate to be 0.6 mM. Km value for ATP was estimated to be about 0.2 mM, and an inhibition was observed at higher concentrations of ATP than 1 mM (Fig. 3B).

Molecular and Regulatory Properties

The molecular wight of the enzyme was estimated to be 64,000 from the experiments using Sephadex G-100 gel filtration (12). This value was identical with that of *E. coli* B (5), which was estimated by the method of Sephadex G-150 gel filtration and of sucrose density gradient centrifugation. No report on the subunit structure of the enzyme has appeared.

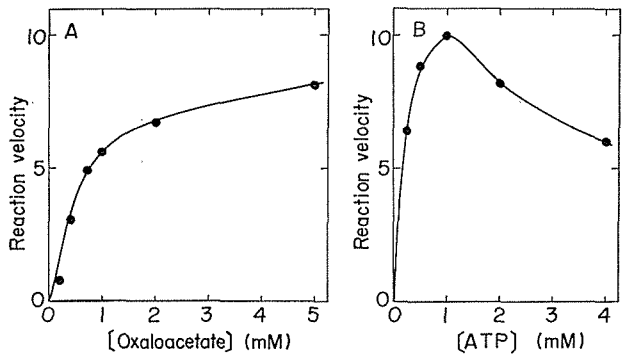

Fig. 3. Effects of oxaloacetate and ATP on the decarboxylation reaction of PEP carboxykinase. The reaction mixture contained 10 μg protein of the enzyme. Experimental conditions were as described in "Materials and Methods" except for the use of substrate concentrations as indicated in the abscissa. Reaction velocity was expressed as nmoles of PEP formed per min.

The enzyme activity was inhibited by SH-blocking reagents—*p* mercuribenzoate and *N*-ethylmaleimide, as in the case of the yeast enzyme (13). Dilution of the enzyme preparation with Tris-HCl buffer, pH 7.5, resulted in a drastic decrease in the enzyme activity. If DTT at a concentration of 0.1 mM or more was present in the buffer for dilution, a complete protection against the inactivation due to dilution was observed. These results indicate that the enzyme from *E. coli* W contains essential SH group(s) for the activity.

Since PEP carboxykinase is one of the key enzymes in gluconeogenesis, it seems likely that the enzyme is presumed to be a regulatory one. Studies on the control of the enzyme level by several groups of investigators including us (2–4) indicate that the enzyme level in *E. coli* is high in gluconeogenesis and low in glycolysis. As for the control of the enzyme activity, there is no evidence supporting that PEP carboxykinase of microbial origin has allosteric effector(s), except for the *E. coli* B enzyme which is regulated with NADH in an allosteric manner (5). So far as examined in a direction of decarboxylation reaction, the *E. coli* W enzyme was not significantly inhibited by NADH. This result is in conflict with the data of the *E. coli* B enzyme whose activity was assayed by determining oxaloacetate by the coloration with Azoene fast violet B. However, NADH (0.5 mM—1 mM) directly inhibited the coloration of oxaloacetate, whereas NAD^+ did not inhibit in a range of concentration of oxaloacetate from 0.05 to 0.5 mM (not shown). Therefore, apparent inhibition of the enzyme by NADH observed by Wright and Sanwal might be due to the inhibition of color development by NADH.

The activity of *E. coli* W enzyme was unaffected by some metabolites, such as glucose 6-phosphate, fructose 1,6-bisphosphate, succinate, aspartate, citrate, 5′-AMP, and 3′,5′-cyclic AMP.

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