Note

Properties of Phosphoenolpyruvate Carboxykinase of an Extreme Thermophile, Thermus thermophilus HB 8

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It has been reported by Yoshida et al.1-4) that phosphoenolpyruvate (PEP) plays an important role in the regulation of the Embden-Meyerhof pathway of an extreme thermophile, Thermus thermophilus HB 8,5,6) The thermophile phosphofructokinase (EC 2.7.1.11) is inhibited by PEP, while the fructose 1, 6-bisphosphatase (EC 3.1.3.11) is activated by PEP. These two enzymes were considered to determine the direction of this pathway in T. thermophilus, depending on the in vivo concentration of PEP.

The thermophile PEP carboxykinase (EC 4.1.1.49) is supposed to function in the metabolic conversion of oxalacetate to PEP in the cells, because T. thermophilus grows well on casamino acids.6) PEP carboxykinases from many organisms are not regulatory enzymes, but induced under gluconeogenetic conditions.7) Only one exception reported is the Escherichia coli enzyme, which is allosterically inhibited by NADH.8) To elucidate the regulatory mechanism by PEP, PEP carboxykinase was partially purified from T. thermophilus and its properties were studied.

T. thermophilus was grown at 75°C in a medium containing 0.8% polypeptone and 0.4% yeast extract as described previously.9) The cells harvested (200 g) were suspended in 400 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl₂, 100 mM KCl, 0.2 mM EDTA, and 0.1 mM dithiothreitol, and disrupted by sonic oscillation at 10 kHz for 5 min. The supernatant fraction was obtained by centrifugation at 75,000 x g for 4 hr with a Hitachi Model 55P ultracentrifuge. All subsequent operations were performed in a cold room. The protein fraction precipitated between 30 and 50% saturation with ammonium sulfate was collected by centrifugation, and dissolved in 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed against the same buffer, and applied to a column (5 x 45 cm) of DEAE-cellulose. The proteins adsorbed were eluted with 3.0 liters of the same buffer containing a linear gradient of KCl from 0 to 0.5 M (Fig. 1). The active fractions were combined, and used as an enzyme preparation (yield, 32%; specific activity, 1.06 units/mg). Neither pyruvate kinase (EC 2.7.1.40) nor PEP carboxylase (EC 4.1.1.31) activity was detected in the sample purified 12-fold.

The enzyme activity of the forward reaction (PEP formation) was assayed at 30°C by coupling with pyruvate kinase and lactate dehydrogenase. Thus the rate of PEP formation was determined by monitoring the decrease in absorbance at 340 or 375 nm (NADH oxidation) with a spectrophotometer (Gilford Model 240 or UVIDEC-2 of Japan Spectroscopic Co.). The reaction mixture (0.475 ml) consisted of 100 mM Tris-HCl buffer (pH 7.5), 2 mM ATP, 2 mM MnCl₂, 2 mM MgCl₂, 30 mM KCl, 0.24 mM NADH, 1.2 units of pyruvate kinase, and 5.5 units of lactate dehydrogenase. After the addition of 25 μl of freshly prepared oxalacetate solution (final concentration, 1 mM) to the reaction mixture, the decrease in absorbance was recorded for about 1 min; the enzyme solution (below 10 μl) was added to the reaction mixture, and the decrease was recorded again. The difference in the rates of NADH oxidation corresponds to the true PEP carboxykinase activity. The enzyme activity of the reverse reaction (oxalacetate formation) was also assayed at 30°C spectrophotometrically. The reaction was initiated by the addition of the enzyme solution

![FIG. 1. DEAE-Cellulose Column Chromatography.](image-url)
(below 10 μl) to the reaction mixture (0.5 ml) consisting of 100 mM imidazole-HCl buffer (pH 6.2), 2 mM PEP, 2 mM ADP, 100 mM NaHCO₃ (freshly prepared), 1 mM MnCl₂, 0.12 mM NADH, and 16.5 units of malate dehydrogenase. The proportional relationships between velocity and enzyme concentration were observed for both assay methods. The incorporation of ¹⁴CO₂ into oxalacetate was observed in the preliminary experiment according to the method of Lane et al.¹⁰ with a modification.

The optimum pH of the PEP formation was 6.5 in imidazole-HCl or 7.5 in Tris-HCl buffer. This enzyme was extremely thermostable as shown in Fig. 2, and hence the enzyme is considered to function at higher temperatures like other enzymes of the thermo phile.¹¹⁻¹⁢⁴,¹⁰⁻¹⁵ In the PEP-forming reaction, a Michaelis-Menten kinetics was observed for oxalacetate (Kₘ = 0.03 mM) or ATP (Kₘ = 0.08 mM). In the oxalacetate-forming reaction, hyperbolic responses with slight substrate inhibitions were observed for both PEP (Kₘ = 0.04 mM) and ADP (Kₘ = 0.04 mM). These Kₘ values are comparable with those for the counterpart enzyme from other organisms.⁷ The Kₘ for bicarbonate was greater than 100 mM, and is at least one order greater than that for the pig liver or yeast enzyme.⁶ The carboxylation of PEP by the thermo phile enzyme is considered to proceed very slowly or not in vivo for the following reasons: first, the enzyme required higher concentrations of bicarbonate as compared with the estimated intracellular level; second, the rate of PEP formation was about 8 times as fast as that of oxalacetate formation.

The effect of various metabolites on the enzyme activity was examined. The enzyme was neither activated nor inhibited when the enzyme activity of PEP formation was assayed in the presence of the following metabolites (1 mM): glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1, 6-bisphosphate, dihydroxyacetone phosphate, d-glyceraldehyde 3-phosphate, glycerol 3-phosphate, glyceral 3-phosphate, glycerate 2-phosphate, AMP, CoA, acetyl-CoA, citrate, isocitrate, 2-ketoglutarate, succinate, fumarate, and malate. The enzyme activity was also unaffected by the addition of low (0.12 or 0.24 mM) or high concentration (1.2 mM) of NADH into the reaction mixture containing the following substrates: 0.2 mM oxalacetate and 0.1 mM ATP (PEP formation); 0.1 mM PEP, 0.1 mM ADP, and 50 mM NaHCO₃ (oxalacetate formation).

As will be described elsewhere, PEP carboxykinase activity per g of protein in the crude extract did not change when the T. thermophilus cells were grown in a medium containing 0.5% glucose and half the concentrations of polypeptone and yeast extract. These findings led us to the conclusion that the T. thermophilus PEP carboxykinase is neither a regulatory enzyme nor an inducible one, and the main function is catalyzing the synthesis of PEP as part of the gluconeogenetic pathway like the enzyme from many other organisms, except it needs higher temperatures. As will be described elsewhere, the T. thermophilus pyruvate kinase shows an allosteric interaction with PEP, and, together with phosphofructokinase and fructose 1, 6-bisphosphatase, serves as one of the key enzymes in glycolysis-gluconeogenesis. Details of the regulatory mechanism by PEP will be described in a subsequent paper.

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REFERENCES

2) M. Yoshida and T. Oshima, ibid., 45, 495 (1971).