Regulation of Pyruvate Kinase from Propionibacterium shermanii

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Abstract. Pyruvate kinase from Propionibacterium shermanii was shown to be activated by glucose-6-phosphate (G-6-P) at non-saturating phosphoenol pyruvate (PEP) concentrations but other glycolytic and hexose monophosphate pathway intermediates and AMP were without effect. Half-maximal activation was obtained at 1 mM G-6-P. The presence of G-6-P decreased both the PEP_{0.5V} and ADP_{0.5V} values and the slope of the Hill plots for both substrates. The enzyme was strongly inhibited by ATP and inorganic phosphate (Pi) at all PEP concentrations. At non-saturating (0.5 mM) PEP, half-maximal inhibition was obtained at 1.8 mM ATP or 1.4 mM Pi. The inhibition by both Pi and ATP was largely overcome by 4 mM G-6-P. The specific activity of pyruvate kinase was considerably higher in lactate-, glucose- and glycerol-grown cultures than that of the enzyme catalysing the reverse reaction, pyruvate, phosphate dikinase. It is suggested that the activity of pyruvate kinase in vivo is determined by the balance between activators and inhibitors such that it is inhibited during gluconeogenesis while, during glycolysis, the inhibition is relieved by G-6-P.

Key words: Pyruvate kinase — Propionibacterium — Glycolytic regulation.

The propionic acid bacteria can meet their energy requirements by anaerobic fermentation of lactate via a well-established sequence of reactions (Allen et al., 1964). Gluconeogenesis from lactate in these bacteria involves a pyruvate, phosphate dikinase (Evans and Wood, 1968) and a pyrophosphate-dependent phosphofructokinase (O'Brien et al., 1975). In view of the low level of ATP-dependent phosphofructokinase and of fructose bisphosphatase in Propionibacterium shermanii it has been suggested that the pyrophosphate-dependent phosphofructokinase functions in both glycolysis and gluconeogenesis (O'Brien et al., 1975). No allosteric effectors of the pyrophosphate-dependent phosphofructokinase have been found and the reaction is freely reversible (Wood et al., 1977). Independent regulation of glycolysis and gluconeogenesis at this point apparently does not occur as in other organisms. The major control point determining the relative glycolytic and gluconeogenic capacities of the organism is therefore likely to be that between phosphoenolpyruvate and pyruvate. The much higher level of the pyruvate, phosphate dikinase in lactate-grown compared to glycerol-grown P. shermanii strongly suggests a gluconeogenic role for this enzyme (Wood et al., 1977). P. shermanii also possesses an active pyruvate kinase (Wood et al., 1977) but the properties of this enzyme have not been previously reported. Regulation of this enzyme is clearly necessary to prevent futile cycling under gluconeogenic conditions. A possible regulatory mechanism based on a study of the properties of the partially purified enzyme is proposed in this paper.

Materials and Methods

Organism. Propionibacterium shermanii ATCC 9614 was obtained from the Department of Food Science and Nutrition, University of Minnesota.

Enzyme Assays. Pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40) activity was determined by measuring the rate of NADH oxidation at 340 nm in the following standard reaction mixture (total volume 1 ml): 100 mM N-tris (hydroxy-methyl) methylglycine (Tricine)/NaOH buffer (pH 7.5), 0.25 mM NADH, 2 mM ADP, 5 mM PEP, 20 mM MgCl₂, 4 units lactate dehydrogenase and 0.1 ml diluted pyruvate kinase. The pyruvate kinase was diluted in 10 mM Tricine/NaOH buffer (pH 7.5) containing 20 % (w/v) glycerol to obtain concentrations of 20 – 40 µg/ml of the partially purified enzyme. Assays were carried out at 30 °C in a
Unicam SP1800 spectrophotometer. One unit of enzyme activity is defined as that amount of enzyme which gives a rate of PEP utilisation of 1 umole per min under the assay conditions described. To eliminate the lag phase before linear rates were obtained, the enzyme was incubated in the reaction mixture minus PEP for 10 min at 30°C and started by addition of PEP.

Pyruvate, phosphate dikinase (ATP:pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1) activity was determined in crude cell-free extracts by measuring the rate of reaction at 30°C in the direction PEP to pyruvate in a coupled reaction with excess lactate dehydrogenase (South and Reeves 1975). The reaction mixture contained the following components in a total volume of 1 ml: 100 mM Tricine/NaOH (pH 7.5), 6 mM sodium pyrophosphate, 6 mM AMP, 24 mM (NH₄)₂SO₄, 10 mM MgCl₂, 2.5 mM PEP, 0.25 mM NADH, 4 units lactate dehydrogenase, 0.1 ml crude cell extract. The reaction was started with PEP after preincubation in the remaining components for 10 min. The crude cell-free extract was obtained in the same way as the extract for pyruvate kinase purification (see below) including the high speed centrifugation to remove NADH oxidase activity.

Protein. Protein was determined by the Coomassie blue binding assay of Bradford (1976). Occasional comparisons with the Lowry et al. (1951) method were used to establish the validity of the binding assay in the present study.

Enzyme Purification P. shermanii was grown in 301 batches in a New Brunswick 50 fermenter at 30°C in the medium described previously (Pritchard et al., 1977) except that sodium lactate was replaced by glycerol at 10 g/l since the specific activity of the pyruvate kinase on the glycerol medium was at least twice that obtained when cultures were grown on a lactate medium. The medium was gassed with 5% CO₂, 95% N₂. Bacteria were harvested after 30 h growth by centrifugation and washed twice in 10 mM Tricine/NaOH buffer (pH 7.5) (containing 10% glycerol for the second wash). The packed cell mass was stored frozen at -15°C. Thirty liters of medium yielded approximately 60 g wet, packed weight of cells. The volumes specified in the following purification procedure are for a 20 g quantity of frozen cell mass.

Bacteria were thawed, suspended in approximately 70 ml 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol, and disrupted by two passages through an Aminco French pressure cell (38,000 kN/m²). All subsequent steps were carried out at 0—4°C. The hemogenate was treated with deoxyribonuclease (10 µg/ml DNase I) for 1 h and then centrifuged at 27,000 × g for 30 min. The supernatant was recentrifuged at 360,000 × g for 2 h. This was necessary to remove fine particulate matter with a high NADH oxidase activity. Nucleic acids were precipitated from the cell-free extract by drop-wise addition of streptomycin sulphate solution, using 2 ml of a 10% (w/v) solution for every 100 mg of protein. The resulting suspension was allowed to stand for 1 h and the precipitate removed by centrifugation at 27,000 × g for 10 min. The supernatant was dialysed against 10 mM Tris (hydroxymethyl) amino methane (Tris)/HCl buffer (pH 7.5) containing 10% glycerol for 2 h. Solid ammonium sulphate was added to the dialysed supernatant to bring the solution to 40% saturation. The precipitate was removed by centrifugation and the concentration of ammonium sulphate increased to 60% saturation. After 1 h the precipitate was collected by centrifugation and redissolved in 10 mM Tris/HCl buffer (pH 7.5) containing 10% glycerol and dialysed against the same buffer for 2 h. The dialysed sample (25 ml) was applied to a DEAE-Sephadex A-25 column (20 × 4.5 cm) equilibrated with 10 mM Tris/HCl (pH 7.5) containing 20% glycerol and 0.1 M KCl. The column was then washed with 100 ml of this buffer after which the enzyme was eluted using a linear gradient of KCl from 0.1—0.4 M in 10 mM Tris/20% glycerol buffer at a flow rate of 1.5 ml per min. All fractions containing the enzyme at a specific activity greater than 3.4 units/mg were pooled and partially concentrated by ultrafiltration using a XM-50 Diaflo membrane (Millipore Corp.).

The concentrated sample was precipitated by adding solid ammonium sulphate to the solution to give a final concentration of 70%. The precipitate was suspended in a minimum volume (less than 3 ml) of 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol and 0.2 M KCl and applied to a Sephacryl S 200 column (90 × 2.5 cm) equilibrated in the same buffer and run at a flow rate of 6 ml per h. All fractions containing the enzyme at a specific activity greater than 30 units/mg were pooled and dialysed against the Tricine/20% glycerol buffer for 3 h. The enzyme was stored frozen at -15°C without significant loss of activity over a period of 4 weeks. The results of the above purification procedure are summarised in Table 1. The presence of glycerol throughout the purification is essential for stability of the enzyme.

Examination of the partially purified preparation by polyacrylamide gel electrophoresis showed in addition to the main pyruvate kinase band, the presence of one major contaminating protein band and several minor bands. The preparation was free from any detectable fructose bisphosphatase, pyrophosphate-dependent phosphofructokinase, pyruvate phosphate dikinase, carbohydrate-phosphorylase, G-6-P dehydrogenase and G-6-P isomerase, NADH oxidase and adenylate kinase.

Growth on Different Carbon Sources. For measuring enzyme activities in cultures grown on different carbon sources the bacteria were grown in the defined medium of de Vries et al. (1973) but with the casein hydrolysate replaced by a mixture of amino acids as follows (g/l): glutamic acid, 3.0, aspartic acid 0.95; lysine 0.65; cystine 0.56; arginine 0.62; histidine 0.55; valine 0.42; tyrosine 0.64; methionine, glycine, proline, threonine, serine, isoleucine, leucine, phenylalanine, tryptophane, alanine, asparagine, glutamine all at 0.2 g/l. Lactate was added at a concentration of 29 g of a 70% (w/v) solution of sodium DL-lactate per l; glycerol at 4 g/l and glucose at 2 g/l (glucose solution sterilised separately). Cultures were grown at 30°C in 2.5 l of medium in a CC1500 fermenter (L.H. Engineering Co., U.K.) gassed with 5% CO₂, 95% N₂. Bacteria were harvested near the end of the log phase (optical density at 540 nm about 2.0).

Chemicals. Biochemicals were obtained from the Sigma Chemical Co., U.S.A. Except where otherwise specified, ADP, guanosine-5'-diphosphate and NADH were solutions of sodium DL lactate (70% solution) were obtained from British Drug Houses Ltd, U.K. The buffer components, Tricine, Tris, 1,3-bis[tris(hydroxymethyl)methylamino]-propane (Bis-tris propane), 2[N-morpholino]ethane sulphonic acid (MES), and N-2-hydroxyethyl piperezine-N'-2-ethanesulphonic acid (HEPES) were all obtained from Sigma.

The lactate dehydrogenase used in the pyruvate kinase assay was a highly purified preparation from pig muscle (500 units/mg protein) free from any detectable pyruvate kinase. For gel-filtration Sephacryl S-200 was obtained from Pharmacia and for ion exchange chromatography, DEAE Sephadex A-25-120 was obtained from Sigma.

Results

Effects of pH on Enzyme Activity

The optimum pH for enzyme activity was determined in several different buffers. A broad optimum over the range pH 7.0—7.8 was obtained in Tricine, HEPES, Bis-tris-propane and MES buffers. However, activity in Tris buffer had a sharp optimum at pH 8.2 and at pH 7.5 was less than 50% of the activity obtained in the other buffers.