Control of Pyruvate Kinase Activity during Glycolysis and Gluconeogenesis in *Propionibacterium shermanii*

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The concentrations of glycolytic intermediates and ATP and the activities of certain glycolytic and gluconeogenic enzymes were determined in *Propionibacterium shermanii* cultures grown on a fully defined medium with glucose, glycerol or lactate as energy source. On all three energy sources, enzyme activities were similar and pyruvate kinase was considerably more active than the gluconeogenic enzyme pyruvate, orthophosphate dikinase, indicating the need for regulation of pyruvate kinase activity. The intracellular concentration of glucose 6-phosphate, a specific activator of pyruvate kinase in this organism, changed markedly according to both the nature and the concentration of the growth substrate: the concentration (7–10 mM) during growth with excess glucose or glycerol was higher than that (1–2 mM) during growth with lactate or at growth-limiting concentrations of glycerol or glucose. Other glycolytic intermediates, apart from pyruvate, were present at concentrations below 2 mM. Glucose 6-phosphate overcame inhibition of pyruvate kinase activity by ATP and inorganic phosphate. With 1 mM-ATP and more than 10 mM inorganic phosphate, a change in glucose 6-phosphate concentration from 1–2 mM to 7–10 mM was sufficient to switch pyruvate kinase from a strongly inhibited to a fully active state. The results provide a plausible mechanism for the regulation of glycolysis and gluconeogenesis in *P. shermanii*.

INTRODUCTION

The propionic acid bacteria are characterized metabolically by their ability to ferment carbohydrates, polyols and organic acids such as lactate to a mixture of propionate, succinate, acetate and carbon dioxide. The fermentation of carbohydrates occurs mainly via the Embden–Meyerhof glycolytic pathway, except that the ATP-dependent phosphofructokinase is largely or completely replaced by an inorganic pyrophosphate-dependent phosphofructokinase (O'Brien *et al.*, 1975). Unlike the ATP-dependent reaction, the pyrophosphate-dependent phosphofructokinase catalyses a freely reversible reaction, so the same enzyme can also function in the gluconeogenic direction when propionibacteria are grown with lactate or glycerol as an energy source. A consequence of this is that the fructose-bisphosphatase/ATP-phosphofructokinase system which usually serves to regulate the relative metabolic flux through glycolysis and gluconeogenesis is replaced by a single enzyme catalysing a reversible reaction so that independent control of glycolysis and gluconeogenesis apparently does not occur at this point. There are, however, independent reactions catalysing the interconversion of phosphoenolpyruvate and pyruvate: a pyruvate kinase catalysing the glycolytic direction and a pyruvate, orthophosphate dikinase catalysing the synthesis of phosphoenolpyruvate during growth on lactate (Wood *et al.*, 1977).

In a previous paper (Smart & Pritchard, 1979) we showed that the pyruvate kinase of *Propionibacterium shermanii* is subject to allosteric regulation, glucose 6-phosphate functioning as a specific positive effector while inorganic phosphate (P_i) and ATP function

as negative effectors. If it is present at a sufficiently high concentration, glucose 6-phosphate can overcome the inhibition of the enzyme by ATP and P_i . It was suggested that pyruvate kinase could be inhibited under gluconeogenic conditions, e.g. during growth on lactate, by ATP and/or P_i , thus preventing substrate cycling; during growth on glucose or glycerol, however, the increased glucose 6-phosphate concentration would overcome this inhibition and permit active glycolysis.

This paper presents the results of experiments designed to test the operation of this postulated control mechanism *in vivo*. The extent to which changes in the activities of glycolytic and gluconeogenic enzymes might contribute to the control of carbohydrate metabolism has also been investigated.

METHODS

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

Growth medium and conditions. Bacteria were grown in the fully defined medium described previously (Smart & Pritchard, 1979) except that when glycerol was used as the growth substrate, sodium acetate (2 g anhydrous salt l^{-1}) was added to the medium. Acetate is apparently an essential growth requirement, at least for this strain, and since it is not produced by propionibacteria growing on glycerol it must be included in the medium. Both batch and continuous cultures were grown at 30 °C in 2.25 l medium in a CC1500 fermenter (LH Engineering Co., U.K.) stirred at 350 rev. min^{-1} and gassed with N_2/CO_2 (95:5, v/v) (N.Z. Industrial Gases). The gas was passed through a heated copper coil at 350 °C to remove traces of oxygen. The pH was maintained at 6.7 ± 0.1 by automatic addition of 2.5 M-NaOH.

Growth was measured by the A_{540} of culture samples, using a dry weight/turbidity calibration curve to obtain dry weights.

Determination of glucose, glycerol and lactate. Substrate concentrations were determined in samples of the culture supernatant after removing the bacteria by centrifugation at 4 °C (27000 g for 5 min).

Glucose was measured enzymically, essentially according to the method of Slein *et al.* (1950). This is based on the fluorometric estimation of NADPH in a system containing hexokinase, glucose-6-phosphate dehydrogenase, NADP and ATP.

Glycerol was determined colorimetrically by the method of Lambert & Neish (1950).

Lactate was determined as its methyl ester by gas-liquid chromatography using a 10% (w/w) EGSS-X (ethylene succinate-methyl silicone copolymer) on a 100–200 mesh Chromosorb Q column in a Packard model 831 gas chromatograph. The methyl esters were prepared directly from samples of culture supernatant according to the method of Siegel *et al.* (1977). The column was run isothermally at 130 °C with flow rates of 33 ml min^{-1} for N_2 and 400 ml min^{-1} for air, and 35 ml min^{-1} for H_2 for the flame ionization detector. Fumarate was used as an internal standard, added to the samples before preparation of the methyl esters.

Fluorometric determination of metabolite concentrations. Samples of culture (containing 17.5 µg dry wt of cells) were rapidly filtered through 0.8 µm Millipore filters and the filter was immediately transferred to 5 ml 0.9 M-perchloric acid on ice. After 20 min, the perchloric acid was neutralized by addition of 0.23 g potassium carbonate and the extract was centrifuged. The supernatant was stored on ice for analysis. Comparison of the metabolite concentrations obtained by this rapid filtration procedure agreed well with those obtained by very rapid quenching as described by Thomas *et al.* (1979).

Metabolite concentrations were determined using the general fluorometric procedures described by Lowry *et al.* (1971) and by Thompson & Thomas (1977), using an Aminco SPF 500 spectrofluorometer. All assays were carried out at 25 °C in a total volume of 2.5 ml in 50 mM-imidazole/HCl buffer (pH 7.1) using reaction mixtures containing components at the final concentrations given in the following descriptions.

Estimation of glucose 6-phosphate, fructose 6-phosphate and ATP: 10 µM-NADP, 10 mM-KCl, 2 mM-MgCl₂, 2 mM-glucose, 100–250 µl cell extract. Glucose 6-phosphate in the extract was determined first by addition of 10 µg glucose-6-phosphate dehydrogenase. After allowing this reaction to go to completion, fructose 6-phosphate was then determined by addition of 4 µg phosphoglucosomerase. Finally, after 2–3 min, ATP was estimated by addition of 10 µg hexokinase.

Estimation of triosephosphates and fructose 1,6-bisphosphate: 5 µM-NADH, 10 mM-KCl, 2 mM-MgCl₂, 100–250 µl cell extract. The triose phosphates were determined after addition of 15 µg triosephosphate isomerase/glycerophosphate dehydrogenase. After 3–5 min, fructose bisphosphate was determined by addition of 10 µg aldolase.

Estimation of pyruvate, phosphoenolpyruvate (PEP) and ADP: 5 µM-NADH, 40 mM-KCl, 2 mM-MgCl₂, and either 1 mM-ADP (for assay of PEP) or 1 mM-PEP (for assay of ADP), 25–250 µl cell extract. Pyruvate in the

extract was first determined by addition of 2 μ g lactate dehydrogenase and, after 3–6 min, either PEP or ADP was determined by addition of 10 μ g pyruvate kinase.

The concentrations of metabolites are reported as μ mol (mg dry wt of cells)⁻¹. In order to convert these levels to in vivo concentrations of metabolites (for comparison with the concentrations of effectors required to activate or inhibit the purified pyruvate kinase in vitro), a value for cell water volume is required. Attempts to obtain this by the usual methods (Cook *et al.*, 1976) were not successful due to the difficulty of finding a totally non-permeant ¹⁴C-labelled solute. Therefore a value of 1.6 μ l (mg dry wt)⁻¹ was taken, as the average of reported values for six different bacteria, all of which lie in the range 1.3–1.7 μ l (mg dry wt)⁻¹. Even if the actual value for *P. shermanii* lies somewhat outside this range it would not significantly affect the conclusions drawn.

Measurement of enzyme activities. Enzyme activities were determined spectrophotometrically by measuring NADH or NADPH production or consumption either directly or by enzymic coupling to NAD(P)H-consuming or producing reactions. Activities were determined at 30 °C in a Unicam SP 1800 spectrophotometer in a total volume of 1 ml, which contained 0.1 ml of suitably diluted cell extract in reaction mixtures containing substrates, cofactors, coupling enzymes and buffers at the concentrations specified below.

Cell extracts were prepared by passing thick cell suspensions in 10 mM-Tricine [N-tris(hydroxymethyl)-methylglycine]/NaOH buffer (pH 7.5) plus 10% (w/v) glycerol twice through an Aminco French press at 38 MPa. The resulting suspension was centrifuged at 27 000 *g* for 15 min at 4 °C. The supernatant from this was further centrifuged at 225 000 *g* for 30 min to remove fine particulate matter with a high NADH oxidase activity.

Enzyme assay mixtures had the following compositions. *Pyruvate kinase* (EC 2.7.1.40): 2 mM-ADP, 0.25 mM-NADH, 20 mM-MgCl₂, 5 mM-PEP, 3 units lactate dehydrogenase, in 0.1 M-Tricine/NaOH buffer (pH 7.5). *Pyruvate, orthophosphate dikinase* (EC 2.7.9.1): 6 mM inorganic pyrophosphate (PP_i), 6 mM-AMP, 0.25 mM-NADH, 25 mM-(NH₄)₂SO₄, 10 mM-MgCl₂, 2.5 mM-PEP, 3 units lactate dehydrogenase, in 0.1 M-Tricine/NaOH buffer (pH 7.5). *PEP carboxytransphosphorylase* (EC 4.1.1.38): 10 mM-KH₂PO₄, 30 mM-KHCO₃, 12 mM-MgCl₂, 0.1 mM-CoCl₂, 0.25 mM-NADH, 2.5 mM-PEP. The reaction mixture minus PEP was brought to pH 6.5 by bubbling with CO₂ for 15 min. The reaction was then started by addition of PEP. Since very high malate dehydrogenase activity was present in crude extracts of *P. shermanii* it was unnecessary to add additional malate dehydrogenase as a coupling enzyme in this assay mixture. *Pyrophosphate-dependent phosphofructokinase*: 1.5 mM-fructose 6-phosphate, 0.25 mM-NADH, 20 mM-MgCl₂, 1 mM-PP_i, 2 units aldolase, 0.83 units triose phosphate isomerase/glycerophosphate dehydrogenase, in 0.1 M-Tricine/NaOH buffer (pH 7.5). *Aldolase* (EC 4.1.2.13): 0.25 mM-NADH, 2 mM-fructose biphosphate, 0.83 units triosephosphate/glycerophosphate dehydrogenase, in 0.1 M-Tricine/NaOH buffer (pH 7.5). *Glucose-6-phosphate dehydrogenase* (EC 1.1.1.49): 0.25 mM-NADP, 10 mM-MgCl₂, 2 mM-glucose 6-phosphate, in 0.1 M-Tricine/NaOH buffer (pH 7.5). *6-Phosphogluconate dehydrogenase* (EC 1.1.1.44): 0.25 mM-NADP, 10 mM-MnCl₂, 2 mM-6-phosphogluconate, in 0.1 M-Tricine/NaOH buffer (pH 7.5).

One unit of enzyme is defined as that amount catalysing the conversion of 1 μ mol substrate min⁻¹ at 30 °C.

Partial purification of pyruvate kinase. The crude extract prepared as described above was treated with streptomycin sulphate to remove nucleic acids and fractionated by ammonium sulphate precipitation and gel filtration through a Sephacryl S-200 column. The procedures used have been described in detail (Smart & Pritchard, 1979). In the partial purification the DEAE-Sephadex step was omitted. The procedure resulted in a fivefold purification of pyruvate kinase. This relatively crude preparation showed the same kinetic response to the various effectors as the much more highly purified preparation used in the previous study.

Protein. Protein concentration was determined according to the Lowry method.

Chemicals. All substrates, cofactors, enzymes and buffers were obtained from Sigma. Amino acids and inorganic salts were obtained from BDH.

RESULTS

Comparison of metabolite concentrations in batch cultures of P. shermanii grown with different energy sources

Batch cultures of *P. shermanii* were grown in 2.25 l fully defined medium containing glucose, glycerol or lactate at the concentrations indicated in Table 1(a). These concentrations of the three energy sources were chosen to give approximately equal yields of cell dry weight per l of culture, based on the molar growth yield values given by deVries *et al.* (1973). Samples were removed for determination of metabolite concentrations at an *A*₅₄₀ of 1.5, i.e. about half way through the exponential phase of growth. Relatively high residual concentrations of all three substrates remained in the medium at the time of sampling (Table 1a).

Table 1 *Metabolite concentrations in P. shermanii grown under different conditions*

Metabolite concentrations are expressed as nmol ($\mu\text{g dry wt}^{-1}$); the values given are averages of two separate determinations, which agreed to within $\pm 10\%$. The values in parentheses represent concentrations (mM) in the cell assuming a value of $1.6 \mu\text{l cell water (mg dry wt)}^{-1}$. (a) Samples were taken for analysis about half way through the exponential phase ($A_{540} = 1.5$). (b) The dilution rate of 0.04 h^{-1} was equivalent to a growth rate of one half to one third of the maximum growth rate in batch culture. Samples were taken at steady state. The steady-state dry weights with the different energy sources were: (g l^{-1}): glucose, 1.1; glycerol, 1.23; lactate, 1.1. (c) Samples were taken at steady state. The steady-state dry weights were (g l^{-1}): with glycerol, 0.89; with lactate, 0.71.

Energy source	Concn of energy source in medium (mM)			Metabolite concn in cells							
	Dilution rate (h^{-1})	Initial	At time sampled	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-bisphosphate	Triose phosphate	PEP*	Pyruvate	ADP*	ATP
(a) Batch culture											
Glucose	NA	11	6.3	16.2 (10.1)	0.85 (0.53)	0.16 (0.1)	0.45 (0.28)	1.0 (0.61)	4.2 (2.6)	2.4 (1.55)	2.1 (1.3)
Glycerol	NA	42	16.8	13.8 (8.6)	4.0 (2.5)	1.0 (0.6)	0.44 (0.27)	0.16 (0.1)	6.3 (4.0)	1.5 (0.9)	1.7 (1.0)
Lactate	NA	120	41.0	2.65 (1.65)	0.72 (0.45)	0.16 (0.1)	0.37 (0.23)	ND	109 (68)	ND	2.2 (1.4)
(b) Continuous culture at low dilution rate											
Glucose	0.04	11	0.14	3.15 (2.0)	0.92 (0.60)	0.29 (0.18)	0.55 (0.34)	1.4 (0.85)	2.35 (1.5)	1.5 (0.90)	2.35 (1.5)
Glycerol	0.04	42	0.10	4.30 (2.7)	0.95 (0.60)	0.27 (0.17)	0.16 (0.10)	2.10 (1.3)	5.35 (3.35)	1.10 (0.70)	2.20 (1.4)
Lactate	0.04	120	0.50	1.95 (1.2)	0.50 (0.31)	0.16 (0.10)	0.39 (0.24)	ND	9.8 (6.1)	ND	1.70 (1.1)
(c) Continuous culture at high dilution rate											
Glycerol	0.09	42	8.2	12.1 (7.55)	2.65 (1.65)	1.25 (0.81)	0.94 (0.59)	0.2 (0.12)	8.45 (4.50)	1.6 (1.0)	1.8 (1.1)
Lactate	0.11	120	27.0	1.7 (1.05)	0.61 (0.38)	0.29 (0.18)	0.36 (0.22)	ND	41 (25)	ND	1.55 (0.98)

NA, Not applicable; ND, not determined.

* Accurate determination of PEP and ADP was not possible in the presence of a high concentration of pyruvate.

Table 2. *Enzyme activities in P. shermanii grown in continuous culture at low dilution rate*

Continuous cultures were grown at a dilution rate of 0.04 h^{-1} . Enzyme activities are expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. Values given are the average of two separate determinations.

Energy source	Pyruvate kinase	PEP carboxy-transphosphorylase	Pyruvate, orthophosphate dikinase	PP _i -dependent phosphofructokinase	Fructose biphosphate aldolase	Glucose-6-phosphate dehydrogenase
Glucose	0.15	0.069	0.078	0.15	0.40	0.013
Glycerol	0.36	0.20	0.046	0.20	0.44	0.007
Lactate	0.20	0.065	0.083	0.12	0.34	0.009

Glucose 6-phosphate was present at a much higher concentration than any other phosphorylated intermediate of glycolysis. The concentration of glucose 6-phosphate was five to six times higher in glucose- or glycerol-grown cells than in lactate-grown cells. Apart from glucose 6-phosphate and pyruvate the concentrations of other intermediates were very similar in lactate- and glucose-grown cells. In glycerol-grown cells, however, the high glucose 6-phosphate concentration was accompanied by increased concentrations of fructose 6-phosphate and fructose 1,6-bisphosphate.

Pyruvate was the only other glycolytic intermediate present at a concentration comparable to that of glucose 6-phosphate. In contrast to glucose 6-phosphate, the concentration of pyruvate was much higher in lactate-grown cells than in those grown in glucose or glycerol media. The high pyruvate concentration in lactate-grown cells created problems for the accurate fluorometric determination of both PEP and ADP, which depends on measuring the amount of pyruvate formed on addition of pyruvate kinase.

Comparison of metabolite concentrations and enzyme activities in chemostat cultures of P. shermanii

While significant differences in metabolite concentrations were found between cells grown on different energy sources in batch culture, it is possible that these differences may have been due, in part, to differences in growth rate or to the stage of growth at which samples were taken rather than to the nature of the energy source itself. Metabolite concentrations were therefore determined in chemostat cultures of *P. shermanii* using different energy sources but maintained at the same growth rate (Table 1*b*). The activities of certain glycolytic enzymes were also determined to assess the extent to which these were affected by the nature of the energy source used (Table 2). The results shown are for chemostat cultures grown at a dilution rate of 0.04 h^{-1} , i.e. at a growth rate of about a third to a half of the maximum growth rate found in batch cultures.

It is clear that at this low dilution rate, growth was limited by the energy source, since the residual concentrations of glucose, glycerol and lactate in the steady-state cultures were very low. Under these conditions, the large differences in glucose 6-phosphate concentration found in the batch cultures were not apparent: the concentration was only about twice as high in glucose- and glycerol-grown cultures as in lactate-grown cells (Table 1*b*). The concentrations of other intermediates were also very similar, apart from pyruvate which was higher in the lactate-grown cells.

The specific activities of glycolytic enzymes were also very similar in cells grown on the three different energy sources, particularly in lactate- and glucose-grown cells (Table 2). In glycerol-grown cells, the activities of pyruvate kinase and PEP carboxytransphosphorylase were somewhat higher, while the activity of pyruvate, orthophosphate dikinase was lower than in glucose- or lactate-grown cells. These differences, though small, are consistent with those reported previously (Smart & Pritchard, 1979). The apparently higher specific activities of pyruvate kinase and pyruvate, orthophosphate dikinase reported in the earlier paper were due to the use of the Coomassie blue binding assay for protein determination (Bradford, 1976) in the earlier study. This method was found to give significantly lower values for protein concentration than the Lowry method used in the present study.

The large difference between the relative metabolite concentrations (especially that of glucose 6-phosphate) in batch cultures and those in the slow-growing continuous cultures could be due to the difference in the concentrations of the energy source in the medium. High concentrations of glucose and glycerol were present in the medium at the time of sampling in the batch culture experiments (Table 1*a*), while in the continuous cultures these growth substrates were barely detectable (Table 1*b*). Therefore a comparison was made between lactate- and glycerol-grown cells in chemostat cultures using a dilution rate close to the maximum growth rate in order to obtain a substrate-excess condition (Table 1*c*). At the dilution rate used in this experiment, relatively high residual substrate concentrations were present. Under such conditions the relative metabolite concentrations were similar to those obtained in the batch culture experiment and quite different from those obtained in the substrate-limited continuous cultures. In particular, the glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate concentrations were much higher in glycerol-grown cells than in lactate-grown cells.

The results of both the batch and the continuous culture experiments indicate that the concentration of glucose 6-phosphate, the specific activator of pyruvate kinase, was much higher under conditions of active glycolysis (i.e. during growth in the presence of excess glucose or glycerol) than during growth on lactate, when gluconeogenesis must have been occurring. The concentration of ATP, an inhibitor of pyruvate kinase activity, was relatively constant (between 1 and 2 mM) under all growth conditions. This would impose a constant inhibitory effect on pyruvate kinase, which would be reversed under conditions of active glycolysis by the high glucose 6-phosphate concentration. The results are thus consistent with the model proposed for regulation of pyruvate kinase in *P. shermanii* (Smart & Pritchard, 1979).

Changes in glucose 6-phosphate concentration during the growth of batch cultures

In order to define more precisely the relationship between glucose 6-phosphate concentrations in the cell and external substrate concentration, the cellular concentration of glucose 6-phosphate was determined at various times during the growth of batch cultures in glucose and glycerol media (Table 3). Glucose 6-phosphate concentration in the cell decreased during growth as the glucose concentration in the medium dropped, but the most striking feature was the abrupt drop in glucose 6-phosphate concentration from 7.5 mM to 1.9 mM corresponding to a very small change in glucose concentration, from 0.71 mM to 0.51 mM (Table 3). There was little further change in glucose 6-phosphate concentration as the culture entered the stationary phase. The concentrations of glucose 6-phosphate found in the cell during the stationary phase of cultures growing on glucose were similar to those in lactate-grown cells during the exponential phase of growth (Table 1*a, b, c*).

There was a similar relationship between external glycerol concentration and intracellular glucose 6-phosphate concentrations. Again glucose 6-phosphate remained at a relatively high level, around 7.5–10 mM, until the glycerol in the medium was exhausted and then dropped abruptly to around 1–1.3 mM.

Table 3. Relationship between concentration of energy source in the medium and glucose 6-phosphate in the cell during growth of batch cultures of *P. shermanii*

Glucose-grown cultures			Glycerol-grown cultures		
A_{540}	Glucose in medium (mM)	Glucose 6-phosphate in cells [nmol (mg dry wt) ⁻¹]*	A_{540}	Glycerol in medium (mM)	Glucose 6-phosphate in cells [nmol (mg dry wt) ⁻¹]*
1.07	6.4	24.0 (15.0)	1.75	16.8	13.9 (8.7)
1.24	4.3	15.0 (9.4)	1.90	16.0	13.0 (8.1)
1.50	3.2	15.8 (9.9)	2.00	13.5	14.7 (9.2)
1.87	2.3	13.9 (8.7)	2.13	9.8	16.3 (10.2)
2.32	0.71	11.8 (7.4)	2.30	7.6	12.5 (7.8)
2.33	0.51	3.0 (1.9)	2.45	6.2	12.5 (7.8)
2.35	0.26	2.7 (1.7)	2.80	2.5	13.6 (8.5)
2.20	0.16	2.2 (1.4)	2.90	0.1	2.1 (1.3)
			2.90	0.1	1.8 (1.1)

* The figures in parentheses represent the concentrations (mM) in the cell assuming a cell water volume of 1.6 μ l (mg dry wt)⁻¹.

Fig. 1. Effect of inorganic phosphate concentration on activity of a partially purified pyruvate kinase preparation at different glucose 6-phosphate concentrations. Pyruvate kinase activity was measured using the procedure described in Methods, but using 1.0 mM-ADP, 0.5 mM-PEP and 1.0 mM-ATP in all assays plus inorganic phosphate and glucose 6-phosphate at the concentrations indicated. Glucose 6-phosphate concentrations: ●, 0 mM; ○, 1 mM; ■, 2 mM; □, 5 mM; ▲, 10 mM; △, 15 mM. The protein concentration was 0.14 mg per assay.

Regulation of pyruvate kinase activity at *in vivo* concentrations of metabolites.

The activity of pyruvate kinase *in vitro* has been shown to be regulated by the effectors glucose 6-phosphate, ATP and P_i (Smart & Pritchard, 1979), the inhibition of enzyme activity by ATP and P_i being reversed by glucose 6-phosphate if the activator was present at a sufficiently high concentration. A further study of this regulation was therefore carried out with a partially purified pyruvate kinase and using the concentrations of substrates and effectors found in the present study to prevail in the cell under various conditions. Of the substrates and effectors found to influence pyruvate kinase activity *in vitro*, the concentrations of PEP, ADP, ATP and glucose 6-phosphate have been determined in the present study but the concentration of P_i in the cell is unknown. In studying the regulation of pyruvate kinase the concentrations of the substrates, PEP and ADP, were fixed at 0.5 mM and 1.0 mM respectively, and the inhibitor, ATP, at 1.0 mM, while glucose 6-phosphate and P_i were used over a range of concentrations.

In the absence of glucose 6-phosphate, pyruvate kinase was completely inhibited by 1 mM-ATP and 2 mM- P_i (Fig. 1). With 1 mM- and 2 mM-glucose 6-phosphate, the inhibition by

Table 4. *Effect of inorganic phosphate concentration on the cooperativity of glucose 6-phosphate activation of the pyruvate kinase from P. shermanii*

The relationship between glucose 6-phosphate concentration and pyruvate kinase activity was determined at seven different phosphate concentrations using the standard assay system described in Methods.

Inorganic phosphate concn (mM)	Interaction coefficient (n_H) of glucose 6-phosphate binding*
0	0.9
0.5	1.4
1.0	2.0
2.0	2.1
4.0	2.0
8.0	2.4
12.0	3.9

* Interaction coefficients were determined by the method of Endrenyi *et al.* (1975).

1 mM-ATP alone was completely reversed but as the P_i concentration was increased to 12 mM, enzyme activity was progressively inhibited. However, at concentrations of glucose 6-phosphate of 5 mM and above, no inhibition of enzyme activity was found even with 12 mM- P_i . Thus in the presence of 1 mM-ATP and 12 mM- P_i , a change in glucose 6-phosphate concentration from 2 mM to 5 mM is sufficient to switch pyruvate kinase activity from a largely inactive form to a fully active form. This is a consequence of the highly cooperative nature of the interaction of glucose 6-phosphate with pyruvate kinase at high P_i concentrations. The results in Table 4 show that with increasing P_i concentration the interaction of glucose 6-phosphate with pyruvate kinase changed from a hyperbolic relationship in the absence of P_i to a very highly sigmoidal relationship ($n_H = 3.9$) at 12 mM- P_i .

DISCUSSION

The primary aim of this investigation was to test the validity of the model proposed in our earlier paper (Smart & Pritchard, 1979) for the regulation of pyruvate kinase in *P. shermanii*. The need for regulation of this enzyme is evident from the results of the study of the relative activities of some of the enzymes of carbohydrate metabolism (Table 2). Pyruvate kinase activity was similar during growth with both 'glycolytic' and 'gluconeogenic' substrates; on both types of substrate its activity was greater than that of pyruvate, orthophosphate dikinase, which catalyses the gluconeogenic conversion of pyruvate to PEP in this organism (Wood *et al.*, 1977). Clearly, independent regulation of glycolysis is not mediated by induction or repression of distinct glycolytic and gluconeogenic enzymes.

In our previous paper (Smart & Pritchard, 1979) we suggested that inhibition of pyruvate kinase by ATP and/or P_i would prevent substrate cycling during gluconeogenesis, but that when, during growth with glucose or glycerol, pyruvate kinase would be required to be active, the increased concentration of glucose 6-phosphate would overcome this inhibition. The high concentration of glucose 6-phosphate found during growth on glucose or glycerol (except when these were present at growth-limiting concentrations) and the low concentration found during growth on lactate support this idea. The difference in glucose 6-phosphate concentrations, found under the different conditions corresponds closely to the difference required to switch pyruvate kinase activity from a strongly inhibited to a fully active state (Fig. 1). The operation of the proposed mechanism requires that at least 10 mM- P_i be present to inhibit pyruvate kinase under gluconeogenic conditions, since the concentration of ATP (1 mM) is insufficient by itself to cause significant inhibition at the glucose 6-phosphate concentrations found in lactate-grown cells. Although sensitive assay methods are available, it would be difficult to obtain reliable values for the true *in vivo* concentrations of free P_i .

because of the high concentration of this compound in the medium (40 mM) and the possibility of its binding to exchange sites in or on the cell. Harold & Spitz (1975) reported a value of 75 mM- P_i in resting cell suspensions of *Streptococcus faecalis* in a medium containing 10 mM- P_i , so a concentration of at least 10 mM- P_i seems not unreasonably high.

The relative concentrations of the different glycolytic intermediates show some distinctive features in *P. shermanii*. Apart from pyruvate, glucose 6-phosphate is the only one of the glycolytic intermediates determined which occurs at concentrations significantly greater than 1 mM during growth on glucose. The concentration of fructose 1,6-bisphosphate found in *P. shermanii* growing in glucose media is less than 0.2 mM even when the glucose concentration in the medium is quite high. This is in marked contrast to the situation in other bacteria. For example, the concentrations of glucose 6-phosphate and fructose-1,6-bisphosphate in rapidly glycolysing *Streptococcus lactis* are 1–2 mM and 15–25 mM respectively (Thompson & Thomas, 1977; Thompson, 1978). Similar high concentrations of fructose 1,6-bisphosphate have been reported in other lactic acid bacteria during growth on glucose as an energy source (Yamada & Carlsson, 1975; Mizushima & Kitahara, 1964). In *Escherichia coli* growing with glucose, concentrations of 1.85 $\mu\text{mol mg}^{-1}$ (about 0.9 mM) glucose 6-phosphate and 6.6 $\mu\text{mol mg}^{-1}$ (3.3 mM) fructose 1,6-bisphosphate were reported by Lowry *et al.* (1971). This difference between the metabolite profile of *P. shermanii* and that in other bacteria may be related to the replacement of the 'irreversible' ATP-dependent phosphofructokinase by the freely reversible PP_i -dependent phosphofructokinase in *P. shermanii*, which would allow an equilibrium to be established between the intermediates fructose 1,6-bisphosphate, fructose 6-phosphate and glucose 6-phosphate. The reversibility of the pathway between triose phosphate and glucose 6-phosphate is supported by the changes in fructose 1,6-bisphosphate, fructose 6-phosphate and glucose 6-phosphate concentrations as exogenous glycerol concentration changed. As the glycerol concentration changed from limiting to excess, the concentrations of all three intermediates increased proportionately by a factor of three- to fourfold.

However, the data on the ratio of glucose 6-phosphate to fructose 6-phosphate in the glucose-grown cultures are not easily explained. The glucose 6-phosphate/fructose 6-phosphate ratios in lactate-grown and in glycerol-grown cells were approximately 4:1, a value similar to that found in other systems, e.g. in cerebral cortex tissue (Rolleston & Newsholme, 1967), in *Streptococcus lactis* (Thompson & Thomas, 1977) and in *Thermus thermophilus* (Yoshizaki & Imahori, 1979), and not greatly different from the value of 2.2:1 expected from the equilibrium constant for the phosphoglucisomerase reaction. However, in glucose-grown cells, the glucose 6-phosphate/fructose 6-phosphate ratio was 19:1. In two other experiments in which metabolite concentrations were determined in batch cultures (results not given), the glucose 6-phosphate/fructose 6-phosphate ratios were found to be 13:1 and 18:1 in glucose-grown cells. These very high ratios suggest either that the properties of the phosphoglucisomerase are unusual or that glucose 6-phosphate is formed very rapidly when cultures are grown on glucose.

As well as its role in controlling the relative flux through glycolysis and gluconeogenesis, the regulation of pyruvate kinase activity may also be important in determining the relative amounts of PEP metabolized via the pyruvate kinase reaction, which generates ATP, and the PEP carboxytransphosphorylase reaction (Siu & Wood, 1962) in which PEP is carboxylated to oxaloacetate and PP_i is generated. This latter reaction results in the accumulation of succinate rather than propionate (Wood *et al.*, 1977) since the transcarboxylase reaction, essential for propionate formation, is by-passed. We have found an increased concentration of succinate among the products of glucose fermentation (results not presented) as cultures enter stationary phase or in glucose-limited continuous culture, i.e. under conditions where glucose 6-phosphate concentration is low and pyruvate kinase activity would therefore be decreased. The metabolic significance of regulation of this branch point is not clear but it may be related to the PP_i -generating function of the carboxytransphosphorylase.

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