Biochimica et Biophysica Acta, 438 (1976) 90—101

BBA 67804

PURIFICATION AND PROPERTIES OF PYRUVATE KINASE FROM *STREPTOCOCCUS LACTIS*

VAUGHAN L. CROW and GRAHAM G. PRITCHARD

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North (New Zealand)

(Received November 21st, 1975)

Summary

The pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) of *Streptococcus lactis* C10 is activated by fructose 1,6-diphosphate (Fru-1,6-P_2), activity being a sigmoidal function of activator concentration. The $FDP_{0.5v}$ (Fru-1,6-P_2 concentration giving half-maximal velocity) is markedly increased in the presence of low concentrations of inorganic phosphate; 1 mM phosphate increases the $FDP_{0.5v}$ value 6-fold. Although the intracellular level of Fru-1,6-P_2 (12—18 mM) in exponentially growing cells on the medium used is much greater than the $FDP_{0.5v}$ for pyruvate kinase (0.2 mM) as determined in triethanolamine · HCl buffer, a much higher Fru-1,6-P_2 concentration may be required to activate the enzyme in vivo to overcome phosphate inhibition. Tris and maleate also inhibit the enzyme. At low concentrations of Fru-1,6-P_2 (0.1 mM), reaction rate is a sigmoidal function of both phosphoenolpyruvate and adenosine diphosphate (ADP) concentrations; at near saturating concentrations of activator (1 mM) the response to varying ADP is hyperbolic while the response to varying phosphoenolpyruvate becomes much less sigmoidal. The affinity for both substrates (especially phosphoenolpyruvate) is also increased by increasing the concentration of Fru-1,6-P_2. The affinity of the enzyme for guanosine disphosphate (GDP) is 12—13 times that for ADP under the assay conditions used. The *Streptococcus lactis* pyruvate kinase has a molecular weight of 240 000 with a subunit molecular weight of 60 000.

Introduction

Collins and Thomas [1] reported that the pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase EC 2.7.1.40) from *Streptococcus lactis* ML3 was acti-

Abbreviations: $FDP_{0.5v}$, fructose 1,6-diphosphate concentration giving half-maximal velocity; $PEP_{0.5v}$, phosphoenolpyruvate concentration giving half-maximal velocity.

vated by fructose 1,6-diphosphate (Fru-1,6-P_2). In this respect, it resembles pyruvate kinases from many different mammalian and microbial sources which have been found to be activated by Fru-1,6-P_2 [2—5]. In *Escherichia coli* there exist two distinct pyruvate kinases [6] only one of which is activated by Fru-1, 6-P_2. A similar situation was reported by Wittenberger et al. [7] for *Streptococcus faecalis* in which two forms of pyruvate kinase, designated pyruvate kinase I and pyruvate kinase II were found. Pyruvate kinase I was not activated by Fru-1,6-P_2, but was slightly stimulated by adenosine 5-monophosphate. Pyruvate kinase II, on the other hand, was almost totally dependent on Fru-1,6-P_2 for activity. Preliminary investigation of pyruvate kinase II suggested that it was a very much smaller molecule (molecular weight 100 000) than the Fru-1, 6-P_2-activated pyruvate kinases from *E. coli* and mammalian sources which are apparently tetrameric enzymes with a molecular weight of about 240 000 [3,4, 8].

The aims of the present investigation were, firstly, to purify and characterise the pyruvate kinase from *S. lactis* more fully to enable a comparison to be made with other microbial pyruvate kinases and, secondly, to compare the Fru-1,6-P_2 activation of the purified pyruvate kinase from *S. lactis* C10 with that of the lactate dehydrogenase of the same organism. The lactate dehydrogenase of *S. lactis* C10, like that of other streptococci [9—11], requires Fru-1,6-P_2 for activity and the regulatory properties of this enzyme have been extensively studied (Crow and Pritchard, unpublished). We have therefore a rather unusual situation in which the activities of two consecutive enzymes of a pathway are controlled by the same activator.

Materials and Methods

Organism. S. lactis C10 was obtained from the Dairy Research Institute, Palmerston North.

Chemicals and Reagents. All substrates and activators were obtained from the Sigma Chemical Co., St. Louis, M. Adenosine 5′-diphosphate (ADP) and guanosine 5′-diphosphate (GDP) were the disodium salts, phosphoenolpyruvate, the trisodium salt and nicotinamide adenine dinucleotide reduced form (NADH), the sodium salt. D-Fructose 1,6-diphosphate was the tetrasodium salt (Sigma Grade 98—100%). The tetracyclohexyl ammonium salt should not be used as this cation inhibited enzyme activity. The lactate dehydrogenase (EC 1. 1.1.27) used in the pyruvate kinase assay was the rabbit muscle type II, a suspension in 2.1 M $(NH_4)_2SO_4$ (10 mg protein/ml, 815 Sigma units/mg). KCl and $MgCl_2 \cdot 6H_2O$ and triethanolamine were analar grade reagents from British Drug Houses. $MgSO_4$ should not be used, as the enzyme is inhibited by sulphate [1]. For gel filtration, Bio-gel A 0.5 m, 100—200 mesh was obtained from Bio-Rad laboratories and for ion-exchange chromatography DEAE-cellulose, Whatman DE-32 (Microgranular) was used.

Pyruvate kinase assay. Pyruvate kinase activity was estimated by measuring the rate of NADH oxidation at 340 nm in a coupled reaction with excess lactate dehydrogenase added to the reaction mixture. The standard assay mixture contained, in a total volume of 3 ml: 80 mM triethanolamine · HCl buffer (pH 7.5), 1 mM phosphoenolpyruvate, 3.3 mM ADP, 1 mM Fru-1,6-P_2, 13.3 mM

KCl, 3.33 mM $MgCl_2$, 0.167 mM NADH, 20 units dialysed lactate dehydrogenase and 0.1 ml of diluted pyruvate kinase. The pyruvate kinase was diluted in 20% (w/v) glycerol at 0°C to obtain protein concentrations of 20—100 μg protein/ml.

Routine assays during enzyme purification were carried out at room temperature using a Unicam SP 800 spectrophotometer. Kinetic studies were carried out at 25°C using a Beckman ACTA-3 spectrophotometer. One unit of enzyme activity is defined as that amount of enzyme which gives a rate of phosphoenolpyruvate utilisation of 1 μmol per min under the assay conditions described.

Protein. Protein was determined by the method of Lowry et al. [12].

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was carried out according to the procedures of Gabriel [13]. Gels consisting of 7% acrylamide and 0.18% *N,N'*-methylenebisacrylamide (pH 8.9) were used. The gels were run in Tris/glycine buffer (pH 8.3) consisting of 3 g Tris, 14.4 g glycine per l of deionised, distilled water. Protein was detected by staining in 0.05% Amido black in 7% acetic acid for 1 h and then destaining in 7% acetic acid overnight. The gels were treated to reveal pyruvate kinase activity in the following way. Immediately after running the gels were washed in 1.1 M triethanolamine · HCl buffer (pH 7.5) for 5 min. They were then placed in an activity mixture containing 2.5 mM NADH, 3 mM phosphoenolpyruvate, 10 mM ADP, 20 mM Fru-1,6-P_2 and 20 units Sigma lactate dehydrogenase in 100 mM triethanolamine · HCl buffer (pH 7.5). The gels were left in this activity mixture for 1 h in the dark at room temperature, and then quickly washed twice in distilled water. The washing was completed in 30 s and the gels were then transferred to a staining mixture containing 5 mg nitro-blue tetrazolium and 2 mg phenazine methosulphate in 8 ml of 100 mM triethanolamine · HCl buffer (pH 7.9). The stain was allowed to develop for 10—20 min in the dark and the gels were then washed three times in 7% acetic acid in the dark. The pyruvate kinase activity region showed as a clear zone against a dark background of reduced nitro-blue tetrazolium.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn [14] using 10% acrylamide gels. Protein was detected by staining with Coomassie brilliant blue. Subunit molecular weight was determined by comparison of the mobility of the pyruvate kinase protein band with those of standard proteins of known molecular weight [14]. The standard proteins used were: bovine serum albumin (Fluka), catalase (bovine liver, Sigma), glutamate dehydrogenase (type I, bovine liver, Sigma), ovalbumin (Sigma), aldolase (rabbit muscle, Sigma), lactate dehydrogenase (rabbit muscle type II, Sigma), carboxypeptidase (bovine pancreas, Sigma).

Results

Enzyme purification. S. lactis C10 was grown in the medium of Jago et al. [15] at 30°C. Cultures were grown without aeration in 5-l Erlenmeyer flasks containing 3 l of medium. The pH was maintained at between 6.0 and 6.5 by periodic addition of 2.5 M NaOH during growth. The cells were harvested near the end of the logarithmic phase of growth by centrifugation at 5500 × *g* for

15 min at 0°C and washed three times in 0.005 M phosphate buffer (pH 7.0) containing 1% NaCl. The washed cells were stored frozen for no longer than 16 h before disruption. Storage of the frozen cells for even a few days appeared to render the partially purified enzyme unstable and this trend became more noticeable with longer periods of storage.

The frozen cells were thawed and suspended in 0.01 M phosphate buffer (pH 7.0) containing 0.05% 2-mercaptoethanol and disrupted by two passages through an Aminco French pressure cell at 5500 lb/inch^2. Unbroken cells and cell debris were removed by centrifugation at 13 000 × *g* at 4°C. All subsequent steps were carried out at 4°C. Nucleic acids were precipitated from the cell-free extract by drop-wise addition of streptomycin sulphate using 3 ml of a 10% (w/v) solution for every 100 mg protein. The resulting suspension was allowed to stand for 2 h before the precipitate was removed by centrifugation at 13 000 × *g*. The supernatant was then dialysed against 0.01 M phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol for 15 h. Powdered $(NH_4)_2SO_4$ was added slowly to the supernatant to bring the solution to 50% saturation and the resulting precipitate removed immediately by centrifugation. The concentration of $(NH_4)_2SO_4$ in the supernatant was then increased to 75% saturation. The precipitate was collected immediately by centrifugation, redissolved in 0.01 M phosphate buffer (pH 6.7) containing 0.1 M KCl plus 0.1% 2-mercaptoethanol and dialysed against the same buffer for 24 h. The dialysed sample was applied to a DEAE-cellulose column (12 × 4.5 cm) pre-equilibrated with 0.01 M phosphate buffer (pH 6.7) containing 0.1 M KCl and 0.1% 2-mercaptoethanol. The column was washed with the same buffer until the absorbance of the eluate at 280 nm had fallen to zero. No pyruvate kinase was found in these fractions. The pyruvate kinase activity was then eluted with 0.02 M phosphate buffer (pH 6.6) containing 0.15 M KCl and 0.1% 2-mercaptoethanol. All fractions containing pyruvate kinase at a specific activity greater than 15 units/mg protein were bulked and concentrated by ultrafiltration using a XM-50 Diaflo membrane. The concentrated solution was dialysed against 0.025 M phosphate buffer (pH 7.3) containing 0.1% 2-mercaptoehtanol for 15 h. An aliquot of the dialysed sample (containing not more than 80 mg total protein in a volume of 7 ml) was loaded on to a 2.5 × 70 cm column of Biogel A 0.5 m (100—200 mesh) pre-equilibrated with 0.025 M phosphate buffer (pH 7.3) containing 0.1% 2-mercaptoethanol. The fractions containing pyruvate kinase with a specific activity greater than 70 units/mg protein were bulked and concentrated to 2 mg protein/ml by ultrafiltration. This solution was then diluted to 1 mg protein/ml with a diluent containing glycerol and $MgCl_2$ such that the enzyme was dissolved in a solution containing 0.005 M phosphate buffer (pH 7.0) containing 50% glycerol (w/v), 0.005 M $MgCl_2$ and 0.1% 2-mercaptoethanol.

The result of the above purification procedure is summarised in Table I. The enzyme was purified overall by 62-fold with a recovery of 29% of the original activity. The purified enzyme was devoid of lactate dehydrogenase activity.

Polyacrylamide disc gel electrophoresis. The purified pyruvate kinase ran as a single major protein band (Fig. 1a) with only very faint minor bands present. Pyruvate kinase activity staining indicated that this major protein band corresponded to the active enzyme. Omission of either phosphoenolpyruvate or ADP from the activity mixture prevented appearance of the activity band,

TABLE I

PURIFICATION OF PYRUVATE KINASE FROM *S. LACTIS* C10

Treatment	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Cell free extract	6400	5280	1.21	1.00	100
Streptomycin sulphate	6500	5000	1.25	1.09	101
$(NH_4)_2SO_4$ 50—75% fraction	4350	1640	2.62	2.18	68
DEAE-cellulose (high specific activity fractions)	2720	124	22.0	18.05	42
Biogel filtration	1850	24.6	75.0	62.0	29

while if Fru-1,6-P_2 was omitted from the mixture, the activity band was only just visible if developed in the stain for 60 min.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis again showed that only trace quantities of impurities were present (Fig. 1b). The mobility of the single major protein band corresponded to a subunit molecular weight of 60 750. Values from six different runs gave values ranging from 59 000 to 62 000.

Equilibrium sedimentation. The molecular weight of the active purified enzyme was determined in a Beckman Model E analytical ultracentrifuge using the equilibrium sedimentation procedure of Chervenka [16]. A value of 235 000 was obtained for the molecular weight.

Effect of pH and buffer composition on pyruvate kinase activity. The optimum pH in 80 mM triethanolamine buffer was 7.5 (Fig. 2). In 80 mM Tris/maleate buffer, pyruvate-kinase activity was greatly inhibited. The effects of maleic acid and Tris on activity were therefore investigated separately. Both components inhibited the enzyme and inhibition was found to be a sigmoidal function of concentration. The data are shown as Hill plots in Fig. 3. Biphasic Hill plots were obtained for both compounds indicating co-operative interaction at high concentrations of the inhibitory ions. 50% inhibition was obtained at a concentration of 22 mM Tris and at 30 mM maleate. When 80 mM phosphate buffer was used as the assay buffer, no pyruvate-kinase activity was detectable. The enzyme was found to be very sensitive to inhibition by phosphate ions with 50% inhibition of activity at 0.65 mM under standard assay conditions. A Hill plot of the inhibition data indicated co-operative binding of phosphate to the enzyme (Fig. 3). Similar inhibition by phosphate was found by Collins and Thomas [1].

Triethanolamine · HCl buffer, pH 7.5, was used in all subsequent studies on the enzyme reported in this paper.

Fructose 1,6-diphosphate activation. The effect of Fru-1,6-P_2 on activity was determined at four different concentrations of phosphoenolpyruvate (Fig. 4). Activity was a sigmoidal function of Fru-1,6-P_2 concentration. Hill plots of the data (Fig. 4b) show that the interaction coefficient is independent of phosphoenolpyruvate concentration over the range studied although the $FDP_{0.5v}$ (Fru-1,6-P_2 concentration giving half-maximum velocity) increased slightly from 0.16 to 0.22 mM as the phosphoenolpyruvate concentration was decreased from 1

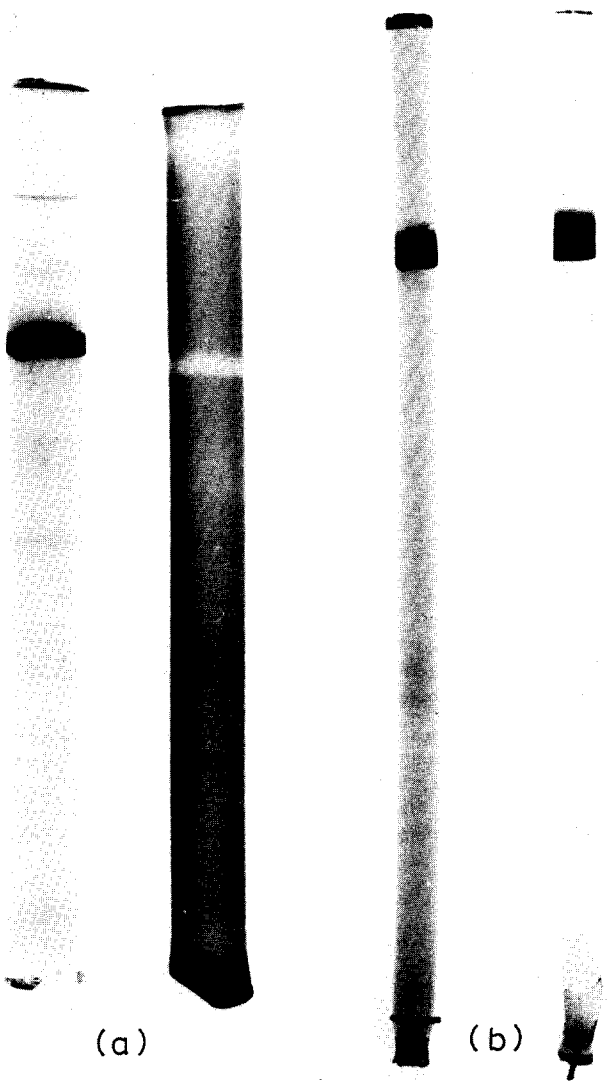

Fig. 1. (a) Polyacrylamide disc gel electrophoresis of purified pyruvate kinase in Tris/glycine buffer (pH 8.3). Details of procedure described in text. 40 μg of purified pyruvate kinase were applied to each gel. The left hand gel stained for protein with Amido black. The right hand gel stained to detect pyruvate kinase activity as described in the text. (b) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The purified pyruvate kinase was treated with sodium dodecyl sulphate and run according to the procedure of Weber and Osborn [14]. Gels were stained with Coomassie brilliant blue to detect protein. The left hand gel was loaded with 30 μg of protein; the right hand gel with 50 μg protein.

to 0.2 mM. The interaction coefficient was also unaffected by varying ADP concentration over a range from 0.3 to 6.67 mM or by increasing the concentration of K^+ and Mg^{2+} from 13.3 mM KCl/3.3 mM $MgCl_2$ to 80 mM KCl/8 mM $MgCl_2$. However, on storage of the enzyme for 1 week at 4°C the interaction coefficient consistently showed a decline from a value of 2.5—2.6 for the freshly purified enzyme to 1.9—2.0. Storage for a longer period caused no further decrease in the co-operativitiy of Fru-1,6-P_2 binding.

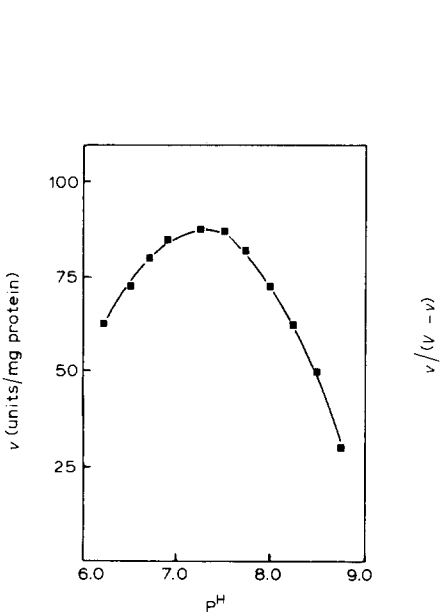

Fig. 2. Effect of pH on pyruvate kinase activity in 80 mM triethanolamine buffer. The reaction mixture for each assay contained (in a total volume of 3 ml): 80 mM buffer, 1 mM phosphoenolpyruvate, 3.3 mM ADP, 1 mM Fru-1,6-P_2, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units dialysed lactate dehydrogenase and 0.1 ml of diluted pyruvate-kinase solution containing 2 μg protein.

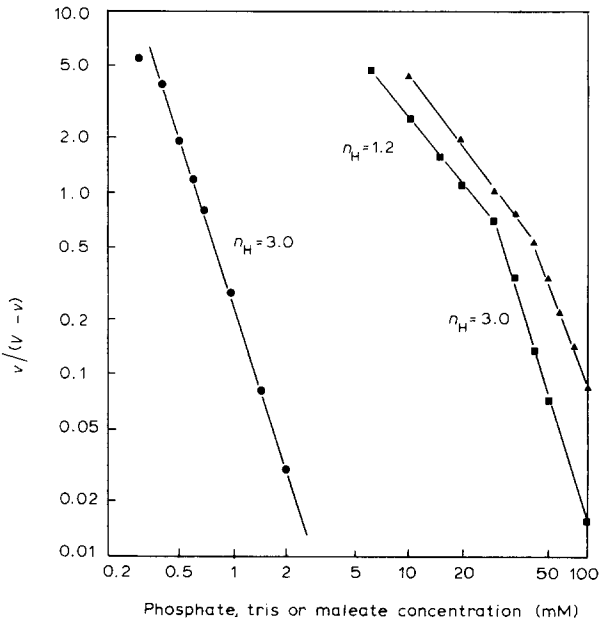

Fig. 3. Inhibitory effect of phosphate (●), Tris (■) and maleate (▲) on pyruvate kinase activity. Data plotted as Hill plots. The reaction mixture for each assay contained (in a total volume of 3 ml): 80 mM triethanolamine · HCl buffer (pH 7.5), 1 mM phosphoenolpyruvate, 3.3 mM ADP; 1 mM Fru-1,6-P_2, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase and 0.1 ml of diluted pyruvate kinase containing 2 μg protein. The pH of the inhibitors was adjusted to 7.5 with triethanolamine · HCl.

Effect of varying phosphoenolpyruvate concentration. The relationship between pyruvate kinase activity and phosphoenolpyruvate concentration was studied at four different Fru-1,6-P_2 concentrations (Figs. 5a and 5b). Unlike Fru-1,6-P_2 activation, which was largely independent of phosphoenolpyruvate concentration, the co-operativity of phosphoenolpyruvate binding was markedly affected by Fru-1,6-P_2 concentration. Decreasing the Fru-1,6-P_2 concentration from 1.0 to 0.15 mM increased both the $PEP_{0.5v}$ values and the n_H values. This same trend has been found for other Fru-1,6-P_2-activated pyruvate kinases including those from *S. lactis* ML3 [1], *E. coli* [4] and the mammalian type-L pyruvate kinase [18]. The $PEP_{0.5v}$ value of the fully activated pyruvate kinase (0.11 mM) agrees fairly well with the value obtained by Collins and Thomas [1] for *S. lactis* ML3 (0.14 mM) but is considerably higher than the $PEP_{0.5v}$ (0.03 mM) for the fully activated pyruvate kinase of *E. coli* [4].

Varying the ADP concentration over a range from 0.4 to 6.67 mM did not affect the cooperativity of phosphoenolpyruvate binding.

Effect of varying ADP concentration. As with other pyruvate kinases [4,17] the *S. lactis* C10 enzyme shows a hyperbolic dependence on ADP concentration at near saturating (1 mM) Fru-1,6-P_2 concentration (Fig. 6) and at several different levels of phosphoenolpyruvate (Fig. 7). However, if the Fru-1,6-P_2 con-

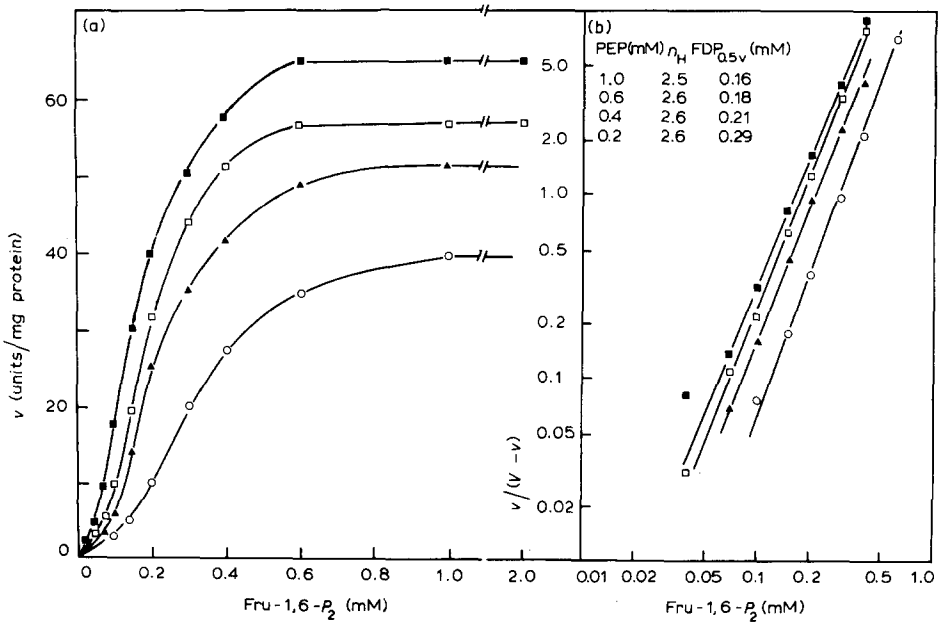

Fig. 4. The relationship between pyruvate kinase activity and Fru-1,6-P_2 concentration at four different concentrations of phosphoenolpyruvate. (a) Reaction velocity as a function of Fru-1,6-P_2, (b) The same data expressed as Hill plots of log $v/(V-v)$ versus log Fru-1,6-P_2 concentration. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine · HCl buffer (pH 7.5), 3.3 mM ADP, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 2.5 μg protein, Fru-1,6-P_2 at the concentrations shown and phosphoenolpyruvate concentrations as follows: ■, 1 mM; □, 0.6 mM; ▲, 0.4 mM and ○, 0.2 mM.

centration is decreased to 0.1 mM the dependence on ADP concentration becomes sigmoidal ($n_H = 1.5$). This property does not appear to have been encountered with other pyruvate kinases.

Michaelis constants for phosphoenolpyruvate and ADP were determined at a fixed, nearly saturating concentration of Fru-1,6-P_2 (1 mM). The K_m value for ADP was 1.2—1.3 mM (Fig. 7) and for phosphoenolpyruvate a value of 0.13 mM was obtained from the secondary plot (Fig. 7, inset). These values are in good agreement with the values found by Collins and Thomas [1] for the pyruvate kinase from *S. lactis* ML3 but the K_m for ADP is considerably higher than that found for the *E. coli* pyruvate kinase (0.24 mM) [4].

Response to guanosine diphosphate (GDP). Waygood and Sanwal [4] reported that the Fru-1,6-P_2-activated pyruvate kinase from *E. coli* could use a variety of other nucleotide diphosphates as phosphate group acceptors of which GDP had the lowest K_m (0.05 mM). GDP was found to be an effective substitute for ADP with the *S. lactis* C10 pyruvate kinase. The relationship between GDP concentration and pyruvate kinase activity (Fig. 8) shows that the K_m for GDP (0.1 mM) is at least 10 times lower than the K_m for ADP (1.2—1.3 mM) under comparable assay conditions.

Effect of phosphate on Fru-1,6-P_2 and phosphoenolpyruvate binding. The effect of varying phosphoenolpyruvate and Fru-1,6-P_2 concentrations on pyruvate kinase activity was investigated in the presence of 1 mM phosphate to ascertain the effect of phosphate ions on the binding of these two ligands. The re-

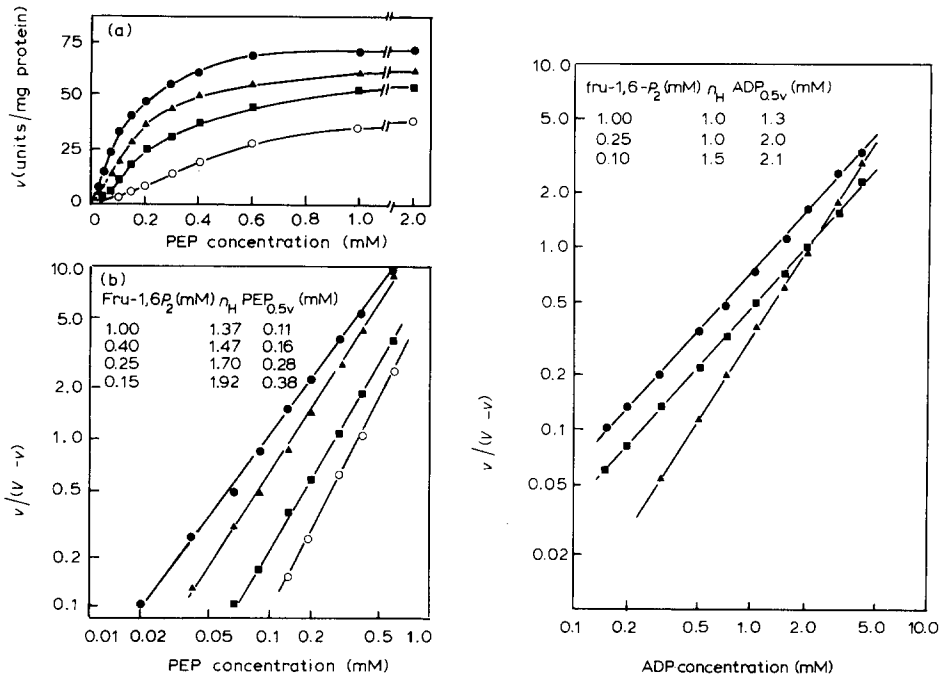

Fig. 5. The relationship between pyruvate kinase activity and phosphoenolpyruvate concentrations at four different concentrations of Fru-1,6-P_2. a, reaction velocity as a function of phosphoenolpyruvate concentration and b, the same data expressed as Hill plots. For each reaction the assay mixture contained (in a total volume of 3 ml): 80 mM triethanolamine · HCl buffer (pH 7.5), 3.3 mM ADP, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml of diluted pyruvate kinase containing 2 μg protein, phosphoenolpyruvate at the concentrations shown and Fru-1,6-P_2 concentrations as follows: ●, 1 mM; ▲, 0.4 mM; ■, 0.25 mM and ○, 0.15 mM.

Fig. 6. The relationship between pyruvate kinase activity and ADP concentrations at three different Fru-1,6-P_2 concentrations and at constant phosphoenolpyruvate. For each reaction the assay mixture contained (in a total volume of 3 ml): 80 mM triethanolamine buffer (pH 7.5), 1 mM phosphoenolpyruvate, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml of diluted pyruvate kinase containing 2 μg protein, ADP at the concentrations shown and Fru-1,6-P_2 at the following concentrations: ●, 1 mM; ■, 0.25 mM and ▲, 0.1 mM.

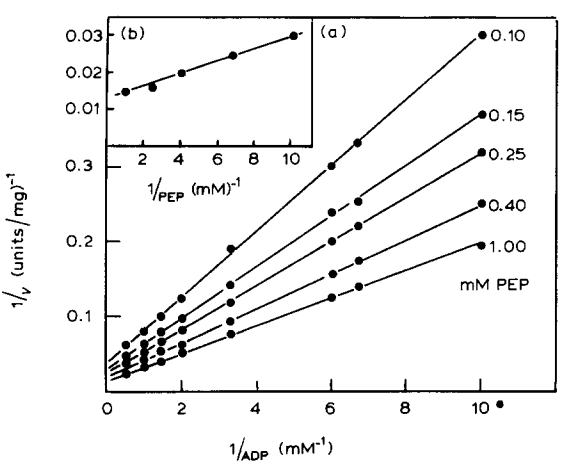

Fig. 7. (a) Lineweaver-Burk plots of $1/v$ versus 1/ADP concentration at five different phosphoenolpyruvate concentrations. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine · HCl buffer (pH 7.5), 1 mM Fru-1,6-P_2, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase and 0.1 ml of diluted pyruvate kinase containing 2 μg protein. ADP and phosphoenolpyruvate are at the concentrations shown on the graph. (b) Secondary plot of the

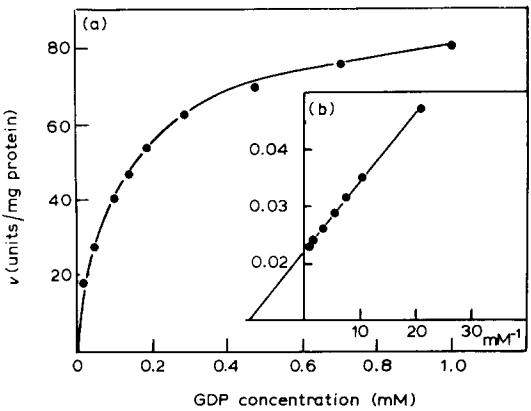

Fig. 8. The relationship between pyruvate kinase activity and guanosine diphosphate concentration. (a) v vs. GDP concentration. (b) Lineweaver-Burk plot of the same data. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine · HCl (pH 7.5), 1 mM phosphoenolpyruvate, 1 mM Fru-1,6-P_2, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase and 0.1 ml of diluted pyruvate kinase containing 2.5 μg protein. GDP is at the concentrations shown.

TABLE II

EFFECT OF 1 mM PHOSPHATE ON KINETIC PROPERTIES OF PYRUVATE KINASE

For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine · HCl buffer (pH 7.5), 3.3 mM ADP, 3.3 mM $MgCl_2$, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml of diluted pyruvate kinase containing 2.5 μg protein and either 1 mM phosphoenolpyruvate and varying Fru-1,6-P_2 or 1 mM Fru-1,6-P_2 and varying phosphoenolpyruvate.

		$PEP_{0.5V}$ (mM)	V (units/mg protein)
Varying phosphoenolpyravate	without phosphate	0.18	73
	with 1 mM phosphate	5.00	80
		$FDP_{0.5V}$ (mM)	V (units/mg protein)
Varying Fru-1,6-P_2	without phosphate	0.16	74
	with 1 mM phosphate	0.93	33

sults are summarised in Table II. The $PEP_{0.5v}$ value was increased from 0.18 to 5.0 mM phosphoenolpyruvate in the presence of 1 mM phosphate although V was not decreased. In the case of Fru-1,6-P_2 the $FDP_{0.5v}$ value increased in the presence of phosphate from 0.16 to 0.93 mM and V decreased considerably. The decrease in V is probably a consequence of the effect of phosphate on phosphoenolpyruvate binding since the phosphoenolpyruvate concentration would have been considerably less than saturating in the presence of phosphate.

Discussion

The present study indicates that, unlike *S. feacalis* [7] and *E. coli* [6] only a single pyruvate kinase appears to exist in *S. lactis*. No other peaks of activity were detected on DEAE-cellulose or Sephadex columns during purification and

the purified pyruvate kinase appears from gel electrophoresis and ultracentrifuge studies to consist of a single enzyme. However, if a second pyruvate kinase had an obligatory requirement for some activator other than Fru-1,6-P_2, it would not have been detected.

The Fru-1,6-P_2-activated pyruvate kinase of *S. lactis* was found to be a tetrameric enzyme, molecular weight about 240 000, with four equal subunits like the pyruvate kinases of *E. coli* [4], rabbit muscle [8] and rat liver [3].

The main aim of the present study was to compare the Fru-1,6-P_2 activation of pyruvate kinase with that of the lactate dehydrogenase in the same strain of *S. lactis* (Crow and Pritchard, unpublished). For both enzymes, the Fru-1,6-P_2 requirement (as measured by the $FDP_{0.5v}$ value) was only slightly affected by varying the concentration of either substrate. However, it has been shown that the Fru-1,6-P_2 requirement for both enzymes is markedly influenced by the chemical nature of the buffering ions used in the assay mixture. Thus the $FDP_{0.5v}$ value for the lactate dehydrogenase was 0.002 mM in triethanolamine buffer, 0.2 mM in Tris/maleate buffer and 4.4 mM in phosphate buffer. With the pyruvate kinase, Tris/maleate and phosphate ions strongly inhibited activity. Phosphate was found to affect the binding of both phosphoenolpyruvate and Fru-1,6-P_2. The pyruvate kinase appears to be even more sensitive to phosphate inhibition than the lactate dehydrogenase. Thus, at 1 mM Fru-1,6-P_2, the concentration of phosphate required to give 50% inhibition of the lactate dehydrogenase was 50 mM whereas for pyruvate kinase the corresponding value was 0.65 mM phosphate. The Fru-1,6-P_2 concentration in exponentially growing cells of lactic streptococci has been shown to be of the order of 15—20 mM (Ref. 1 and Crow and Pritchard, unpublished) which is many times greater than the $FDP_{0.5v}$ value for the both the lactate dehydrogenase (0.002 mM) and the pyruvate kinase (0.16 mM) when these values are determined in triethanolamine buffer. However, these $FDP_{0.5v}$ values may not be relevant to the in vivo situation where phosphate ions may be present at quite high concentrations. Thus in a recent study of phosphate accumulation in *S. faecalis*, Harold and Spitz [18] found that the inorganic phosphate concentration in cell grown on a medium containing 10 mM phosphate was 93 mM. Since 1 mM phosphate increases the $FDP_{0.5v}$ value from 0.16 to 0.93 mM (under standard assay conditions in triethanolamine buffer) it is clear that quite high Fru-1,6-P_2 concentrations could be required for activation in vivo.

Acknowledgement

We are indebted to the late Dr. J.W. Lyttleton for his help in determining the molecular weight by equilibrium sedimentation in the ultracentrifuge.

References

1 Collins, L.B. and Thomas, T.D. (1974) J. Bacteriol. 120, 52—58
2 Taylor, C.B. and Bailey, E. (1967) Biochem. J. 102, 32C—33C
3 Tanaka, T., Harano, Y., Sue, F. and Morimura, H. (1967) J. Biochem. Tokyo 62, 71—91
4 Waygood, E.B. and Sanwal, B.D. (1974) J. Biol. Chem. 249, 265—274
5 Hess, B., Haeckel, R. and Brand, K. (1966) Biochem. Biophys. Res. Commun. 24, 824—831

6 Malcovati, M. and Kornberg, H.L. (1969) Biochim. Biophys. Acta 178, 420—423
7 Wittenberger, C.L., Palumbo, M. and Klein, I. (1973) in The Ninth International Congress of Biochemistry, Stockholm, Abstract 2e, 15, 62
8 Steinmetz, M.A. and Deal, W.C. (1966) Biochemistry 5, 1399—1405
9 Wittenberger, C.L. and Angelo, N. (1970) J. Bacteriol. 101, 717—724
10 Jonas, H.A., Anders, R.F. and Jago, G.R. (1972) J. Bacteriol. 111, 397—403
11 Brown, A.T. and Wittenberger, C.L. (1972) J. Bacteriol. 110, 604—615
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Gabriel, O. (1971) Methods in Enzymology (Jakoby, W.B., ed.), Vol. 22, pp. 565—578, Academic Press, New York
14 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
15 Jago, G.R., Nichol, L.W., O'Dea, K. and Sawyer, W.H. (1971) Biochim. Biophys. Acta 250, 271—285
16 Chervenka, C.H. (1970) Anal. Biochem. 34, 24—29
17 Carminatti, H., Jimenez de Asua, L., Recondo, E., Passeron, S. and Rozengurt, E. (1968) J. Biol. Chem. 243, 3051—3056
18 Harold, F.M. and Spitz, E. (1975) J. Bacteriol. 122, 266—277