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Purification and kinetic properties of pyruvate kinase isoenzymes of *Salmonella typhimurium*

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1. Two forms of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) present in *Salmonella typhimurium* were purified to homogeneity from the same cultures by $(NH_4)_2SO_4$ fractionation and gel filtration, anion-exchange and affinity chromatography. 2. M_r values, subunit structure, amino acid composition and activity and stability conditions were determined for the two forms. 3. Kinetic and regulatory properties of the two purified isoenzymes were studied.

INTRODUCTION

Pyruvate kinase (EC 2.7.1.40) catalyses the formation of pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP, one of the most important steps in the Embden–Meyerhof pathway. Multiple forms of the enzyme have been observed in a wide range of cells, and seem to play distinct physiological roles in metabolism (Seuber & Schoner, 1971; Ibsen, 1977; Hall & Cottan, 1978). In tissues such as liver and kidney and in many bacterial cells, where the processes of glycolysis and gluconeogenesis occur in the same cells, it is important to regulate these mutually opposing sequences, and it seems that regulation of pyruvate kinase activity is significant in this respect.

Two pyruvate kinases in *Escherichia coli* have been well characterized and their kinetic and regulatory properties studied (Malcovati *et al.*, 1973; Waywood & Sanwal, 1974; Waywood *et al.*, 1975; Kotlarz *et al.*, 1975; Somani *et al.*, 1977; Valentini *et al.*, 1979). Their importance has been emphasized with mutants defective in both isoenzymes (Garrido-Pertierra & Cooper, 1977), and evidence of two distinct pyruvate kinase genes was found (Garrido-Pertierra & Cooper, 1983). *Salmonella typhimurium* has a phenotype and genotype almost as well documented as those of *E. coli*, but very poor information has been found about pyruvate kinase activity, and, although there are various systems or reactions potentially capable of transforming PEP to pyruvate, this activity seems to be important in the regulation of carbohydrate metabolism.

In order to gain information on the physiological function of the pyruvate kinase activity in *S. typhimurium*, we have undertaken to purify the isoenzymes to homogeneity, to study the structure and mode of regulation and to elucidate the mechanism of the activation or inhibition by some effectors.

MATERIALS AND METHODS

Organism

The strain utilized was *S. typhimurium* LT-2, obtained from the Department of Biochemistry, University of Leicester, U.K.

Growth conditions and preparation of the cell extracts

The cells were grown aerobically at 37 °C in suitably supplemented minimal medium 63 (Miller, 1972) containing 10 mM-hexose, 20 mM-glycerol or -lactate, or 30 mM-acetate. The growth rate was monitored by measuring the A_{680} of the culture. In the second half of the exponential phase, the cells were harvested, washed with buffer [50 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/2 mM-2-mercaptoethanol/50 mM-KCl], and their wet weight was measured; they were stored at −20 °C. To obtain the crude extract, the cells were suspended in 2 vol. (v/w) of the above buffer. The cell suspension was disrupted by ultrasonic treatment with a Branson B-12 sonifier (70–80 W) at ice temperature for 30 s periods, with cooling for 1 min in between. The suspension was centrifuged at 25000 *g* for 15 min at 4 °C to remove cell debris.

Protein determination

Protein was measured either colorimetrically, with crystalline bovine serum albumin as standard, by the modified Folin method (Lowry *et al.*, 1951), or spectrophotometrically (Warburg & Christian, 1941).

Enzyme assays

For assays in cell extracts, the supernatant solutions were further centrifuged at 150000 *g* for 1.5 h at 4 °C to remove the particulate NADH oxidase activity. Pyruvate kinase was assayed by measuring the decrease in A_{340} at 30 °C in a Beckman model 35 recording spectrophotometer, essentially by the method of Malcovati *et al.* (1973). The reaction mixture contained, in 1 ml: Hepes buffer, pH 6.8 (30 μmol), $MgCl_2$ (5 μmol), NADH (0.15 μmol), ADP (2 μmol), crystalline lactate dehydrogenase (5 μg) and bacterial extract (approx. 40 μg of protein). After measurement of the endogenous rate of NADH oxidation, the reaction was initiated by the addition of PEP (1 μmol). The activity observed in this assay was taken to be due to pyruvate kinase II. The assay was repeated in the additional presence of fructose 1,6-bisphosphate (1 μmol), and the difference in rate between the two assays was assumed to be due to pyruvate kinase I. The ratio of NADH oxidized to

Abbreviation used: PEP, phosphoenolpyruvate

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Table 1. Summary of the purification of pyruvate kinases from *Salmonella typhimurium* LT-2

Data for purification starting with 12 g wet wt. of cells.

			Pyruvate kinase							
			Form I				Form II			
Purification step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/ mg of protein)	Yield (%)	Purification (fold)	Activity (units)	Specific activity (units/ mg of protein)	Yield (%)	Purification (fold)
I. Crude extracts	50	1600	510	0.31	–	–	450	0.28	—	—
II. $(NH_4)_2SO_4$ fractionation	9	720	432	0.60	84	1.8	324	0.45	72	1.6
III. Sephacryl S-200 fractions	22	220	212	0.96	42	3.0	141	0.64	31	2.3
IV. DEAE-Sephacel fractions	21.9 15.0	4.8 4.5	81	16.80	15.8	18.4	45	10.00	10	35.7
V. Blue Sepharose CL6B fractions	7.5 16.0	0.23 0.32	42	180.60	9.2	582	36.8	115.00	8	410

pyruvate formed was assumed to be unity. One enzyme unit is defined as the amount which catalyses the formation of 1 μmol of pyruvate/min, and specific activity is given in enzyme units/mg of protein. Assays under other conditions were as described in the text.

Purification of the enzyme

All the experiments were performed at 4 °C. The purification steps were carried out by the following procedure and are summarized in Table 1. The centrifuged extract prepared from about 12 g of cells was diluted with 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA, 2 mM-2-mercaptoethanol and 50 mM-KCl (extracting buffer) until a solution of approx. 30 mg of protein/ml was obtained. This solution was fractionated by addition of $(NH_4)_2SO_4$, and the proteins precipitated at between 40 and 65% saturation were dissolved in 7 ml of extracting buffer and applied to a column (41 cm × 2.6 cm) of Sephacryl S-200 (superfine grade) equilibrated against extracting buffer. The column was eluted at a flow rate of 22 ml/h at constant pressure, and fractions (each 2.25 ml) were collected. The ten fractions containing the peak pyruvate kinase activity were pooled and applied to another column (30 cm × 2.6 cm) of DEAE-Sephacel previously equilibrated against extracting buffer. A linear gradient of 0.0–0.35 M-KCl in 200 ml of extracting buffer was applied to the column, and fractions (each 2.0 ml) were collected. The first fractions with pyruvate kinase activity (form I) from the DEAE-Sephacel column (Fig. 1), i.e. fractions 52–61, were pooled, dialysed overnight against the extracting buffer and applied to a small column (17 ml) of Blue Sepharose CL 6B. This column was eluted with a linear gradient of 0.0–0.5 M-KCl dissolved in 100 ml of extracting buffer. The other

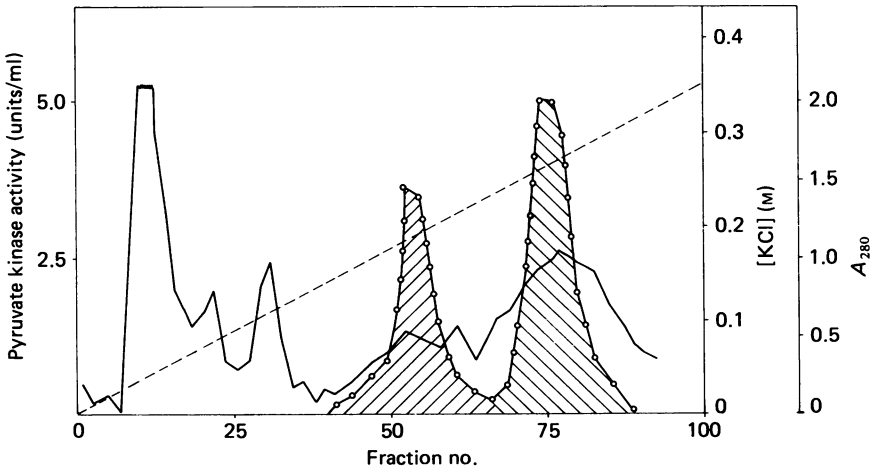

Fig. 1. Profile of elution from DEAE-Sephacel column

The ion exchanger was equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA, 2 mM-2-mercaptoethanol and 50 mM-KCl, and eluted with a gradient of 0.0–0.35 M-KCl in the equilibrating buffer. ○, Pyruvate kinase activity; ——, A_{280} (protein); – – – –, [KCl].

Table 2. Amino acid analysis of form I and II pyruvate kinase from *Salmonella typhimurium* LT-2

Key: —, not detected.

Amino acid	Form I: Residues per subunit of M_r 54000			Form I: Average (extrapolated)	Form II: Residues per subunit of M_r 47000			Form II: Average (extrapolated)	Integer no.	
	24 h*	48 h†	72 h†		24 h*	48 h†	72 h†		Form I	Form II
Lys	37.83	38.40	38.19	38.14	27.51	27.79	28.10	27.80	38	28
His	8.71	8.58	8.98	8.75	4.88	5.10	5.37	5.11	9	5
Arg	19.68	20.07	21.71	20.52	25.56	25.71	25.95	25.74	20	26
Asp	56.30	56.59	57.42	56.77	55.22	55.79	55.96	55.65	57	56
Thr‡	35.68	35.23	34.82	36.15	30.02	29.09	28.81	30.85	36	31
Ser‡	27.41	27.11	26.68	27.70	27.34	26.78	26.37	28.85	28	27
Glu	51.76	50.92	50.77	51.15	35.74	35.93	35.73	35.80	51	36
Pro	14.91	14.84	14.51	14.75	14.86	15.50	15.27	15.21	15	15
Gly	42.18	42.09	42.45	42.24	40.23	40.82	40.63	40.76	42	41
Ala	40.02	40.41	40.51	40.31	49.41	49.89	50.25	49.85	40	50
Val§	38.90	39.16	40.68	40.68	43.10	43.78	44.07	44.07	41	44
Met	16.06	17.43	18.08	17.19	10.96	12.47	12.18	11.87	17	12
Ile§	31.25	32.77	34.11	34.11	22.40	22.86	23.90	23.90	34	24
Leu	39.82	39.90	40.73	40.15	32.72	32.84	34.34	33.30	40	33
Tyr§	6.88	6.07	5.24	6.88	5.08	4.32	4.10	4.69	7	5
Phe	11.33	11.62	12.84	11.93	7.53	7.86	8.70	8.03	12	8
Cys‖	14.06	—	—	—	5.97	—	—	—	14	6
Trp¶				0				0		
Total									501	447

* Average of three analyses.
† Average of two analyses.
‡ Extrapolated to zero time.
§ Maximum values taken.
‖ Determined as cysteic acid.
¶ See the text.

isoenzyme (form II) was obtained by a similar procedure to that described above. In this case, fractions 72–79 (Fig. 1) from the DEAE-Sephacel column were pooled, dialysed and applied to the Blue Sepharose column previously equilibrated against the extracting buffer. The column was eluted with a linear gradient of 0.0–1.0 M-KCl dissolved in 200 ml of extracting buffer.

Determination of M_r and Stokes radius

The gel-filtration column (Sephacryl S-200) and conditions used in the purification were used to determine the M_r of each enzyme and its Stokes radius. Reference markers were Blue Dextran (M_r 2×10^6), β-amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 67000), carbonic anhydrase (M_r 29000) and cytochrome *c* (M_r 12400). It was assumed that all proteins concerned were of the same shape and solvation.

Electrophoresis

The purity of the enzymes was tested by electrophoresis in 7.5%-polyacrylamide gels as described by Davis (1964). The M_r values of the enzyme monomers were calculated by SDS/polyacrylamide-disc-gel electrophoresis (Weber & Osborn, 1969). Reference proteins were: bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), pepsin (M_r 34000), trypsinogen (M_r 24000), β-lactoglobulin subunit (M_r 18400) and lysozyme (M_r 14300).

Amino acid analysis

This was carried out on a Beckman automatic amino acid analyser, model 120B, equipped with a column filled with M70 Beckman resin. The method used was described by Spackman *et al.* (1958). Samples of protein were hydrolysed in 5.7 M-HCl containing 0.1% (v/v) phenol in sealed evacuated tubes for 24, 48 and 72 h at 108 °C. Cysteine was analysed as cysteic acid by the method of Spencer & Wold (1969). Tryptophan and tyrosine were determined independently by the spectrophotometric method of Edelhoch (1967).

Calculation of kinetic parameters

The Hill coefficient (*h*) was calculated from Hill plots (Cornish-Bowden & Koshland, 1975). When sigmoidal kinetics were obtained from plots of initial velocity against substrate concentration, the apparent $V_{max.}$ values were estimated from Scatchard plots.

Chemicals

The sodium salts of AMP, ADP and ATP, the monocyclohexylammonium salt of PEP, EDTA, Hepes, rabbit muscle lactate dehydrogenase (type II), Blue Dextran and electrophoresis materials were from Shandon Southern Products, London N.W.10, U.K. Sephacryl S-200, DEAE-Sephacel and Blue Sepharose CL 6B were from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest purity commercially available.

RESULTS

Purification of the pyruvate kinases I and II

When *S. typhimurium* was grown on different carbon sources (glucose, glycerol, fructose, acetate, lactate and succinate) and the specific activities of the pyruvate kinases were determined, the highest activities were found in cells grown on glycolytic susbstrates. Therefore, from extracts of cells grown in glucose, the pyruvate kinases were purified by fractionatal precipitation with $(NH_4)_2SO_4$ and gel-filtration, anion-exchange and affinity chromatography. The pyruvate kinase activities were separated on the DEAE-Sephacel column and called forms I and II according to their position in the elution profile (Fig. 1). From the Blue Sepharose CL 6B column the pyruvate kinase I was eluted at 0.2 M-KCl, and form II at 0.7 M-KCl. After this step, form I was purified more than 580-fold with a yield of 9.2%, and form II 410-fold with a yield of 8%. The purity of the enzyme was tested by polyacrylamide-disc-gel electrophoresis of samples of the most active fractions, which showed only one band after staining of gels.

M_r values and subunits

The M_r values of the native proteins, estimated by gel filtration, were 220000 ± 10000 and 190000 ± 10000 for forms I and II respectively. Likewise, from the data obtained by gel filtration, the Stokes radius were calculated to be 4.8 and 4.63 nm for pyruvate kinase I and II respectively. The M_r values obtained are in good agreement with those published for pyruvate kinase from *E. coli* (Waywood & Sanwal, 1974; Waywood *et al.*, 1975; Valentini *et al.*, 1979) and are well within the range of M_r values observed for other pyruvate kinases (Giles *et al.*, 1975; Kagda *et al.*, 1979; Abbe & Yamada, 1982).

Electrophoresis of pyruvate kinases in polyacrylamide gel containing SDS gave a single band corresponding to M_r 54000 ± 1000 for form I and 47000 ± 1000 for form II. Like all other pyruvate kinases described so far, the isoenzymes of *S. typhimurium* appear to be tetrameric proteins.

Amino acid composition

Table 2 shows the amino acid analysis of purified pyruvate kinases I and II. The data are expressed as nearest whole number of amino acid residues per subunit molecule. The residue totals are 501 and 447 for form I and II respectively. The absence of tryptophan from both types is in agreement with data for the homologous enzymes from *E. coli* (Waywood & Sanwald, 1974; Somani *et al.*, 1977), yeast (Bornmann & Hess, 1974) and rabbit muscle (Cottam *et al.*, 1969). However, the presence of a tryptophan residue has been described in pyruvate kinase from *Saccharomyces cerevisiae* (Burke *et al.*, 1983) and cat muscle (Muirhead *et al.*, 1986).

Conditions for activity and stability

Both pyruvate kinase isoenzymes showed an absolute requirement for Mg^{2+} or Mn^{2+}, although Mg^{2+} was more effective. When the enzyme activities were measured in the pH range 4.0–8.5 in sodium acetate, Tris/maleate, Hepes and sodium phosphate buffers, both activities were found to increase from pH 4.0 to 6.8 and decline at higher pH values. Form I showed a broader bell-shaped pH curve than did form II. The enzymes were more active at equivalent pH values in Hepes than in other buffers.

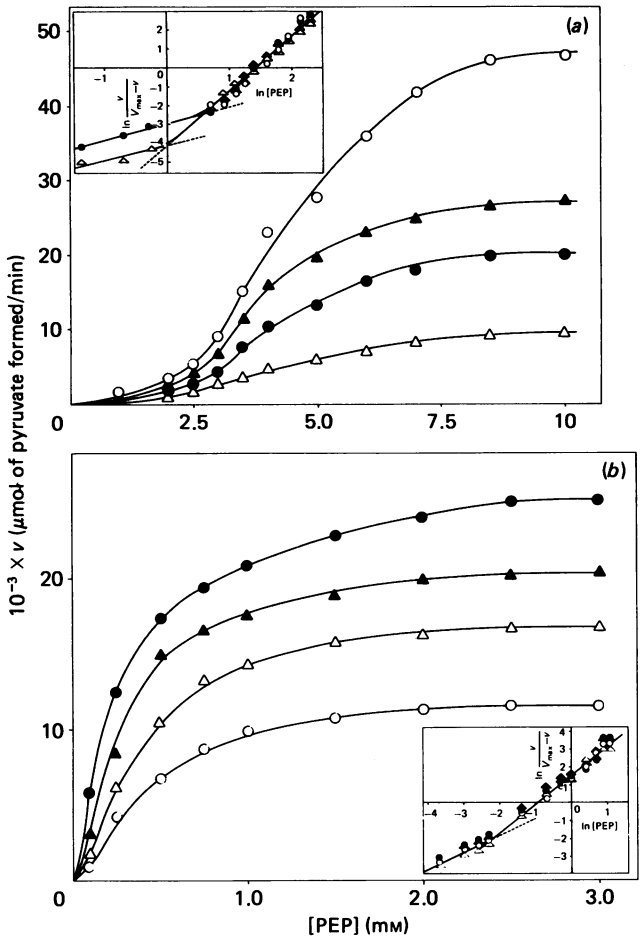

Fig. 2. Kinetic response of the two forms of pyruvate kinase with PEP

The assay mixture contained, in 1 ml: 30 mM-Hepes buffer, pH 6.8, 5 mM-$MgCl_2$, 0.15 mM-NADH, 5 μg of crystalline lactate dehydrogenase, variable PEP concentration and either (*a*) 3 μg of purified pyruvate kinase I and ADP concentration as indicated (△, 0.10 mM; ●, 0.25 mM; ▲, 0.45 mM; ○, 1.00 mM) or (*b*) 4 μg of purified pyruvate kinase II and ADP concentrations as indicated: (○, 0.10 mM; △, 0.25 mM; ▲, 0.50 mM; ●, 1.00 mM). Hill plots of the data saturation for PEP are shown in the insets.

The apparent inhibition of the activities by Tris/HCl and phosphate buffers has been observed in homologous enzymes from rabbit muscle (Ainsworth & Macfarlane, 1975), *Bacillus licheniformis* (Touminen & Bernlohr, 1971) and *Streptococcus* mutants (Abbe & Yamada, 1982).

The stability of the pyruvate kinases was examined, by dialysis against 50 mM-Tris/HCl, 50 mM-Hepes and 50 mM-Tris/maleate buffers in the pH range 5.5–8.0. The proteins were maintained in these solutions and incubated at two temperatures, 25 and 37 °C. Every 15 min, samples were taken and assayed for residual pyruvate kinase activity. Both enzymes showed a broad range of stability between pH 7.0 and 8.0 and independent of the buffer. The inclusion of EDTA, 2-mercaptoethanol, dithiothreitol, glycerol and KCl was found to stabilize the enzyme on storage, being more effective under refrigerated conditions than at room tem-

Table 3. Kinetic parameters of the homotropic interactions of pyruvate kinases with Mg^{2+}

The experiments were carried out at variable Mg^{2+} concentrations (0.01–5.0 mM) and under standard conditions. The enzyme assays for form I contained 3 μg of purified enzyme and those for form II 4 μg.

Isoenzyme	Substrate (constant concn.) (mM)	Substrate (fixed concn.) (mM)	$s_{0.5}$ (mM)	h	$V_{max.}$ (μmol of pyruvate formed/min)
I	PEP 10	ADP 0.25	1.1	2.5	20.5
		ADP 0.50	1.2	2.5	33.0
		ADP 1.00	1.5	2.5	53.0
	ADP 0.5	PEP 3.50	1.5	2.5	14.0
		PEP 10.00	1.8	2.5	33.0
II	PEP 3.0	ADP 0.1	0.78	1.2	23.0
		ADP 1.0	0.90	1.2	25.0
	ADP 0.3	PEP 0.5	0.77	1.2	24.0
		PEP 1.25	1.00	1.2	24.0

perature. Pyruvate kinase I, dissolved in 50 mM-Tris/HCl, did not appreciably lose activity when stored for up to 10 days at 4 °C, or at least 9 months when frozen; values for isoenzyme II, in the same conditions, were 4 days and 4 months respectively.

Effect of temperature

The two purified forms of pyruvate kinase showed different behaviour with temperature: although pyruvate kinase I only lost 12% activity after incubation at 55 °C for 15 min, pyruvate kinase II activity was decreased to zero. A plot of the log of initial velocity against 1/temperature for form I showed two straight lines between 25–41 °C and 41–55 °C, with values of $E_a = 44.2$ and 11.2 kJ/mol respectively. Form II showed one straight line, with a value of $E_a = 43.9$ kJ/mol. The E_a values found for pyruvate kinase II and at low temperature for pyruvate kinase I are similar to those described for the homologous enzyme from fish (Hochachka & Somero, 1971; Gaitan *et al.*, 1983).

Influence of substrate concentration on catalysis by pyruvate kinases

Effect of PEP concentration. Pyruvate kinase I showed sigmoidal kinetics when PEP was the variable substrate at four fixed concentrations of ADP and the other assay conditions were maintained standard. These saturation curves are indicative of a positive co-operativity, with $s_{0.5}$ values between 3.7 and 4.5 mM (Fig. 2*a*). When data were replotted as Hill plots, straight lines were obtained, with Hill coefficient (h) about 3.0, which suggests more than three PEP-binding sites on the enzyme with some degree of interaction between them. When pyruvate kinase II was treated in the same way as isoenzyme I, saturation curves were obtained (Fig. 2*b*) with $s_{0.5}$ values between 0.24 and 0.40 mM for the four concentrations of ADP assayed. The value of the Hill coefficient was 1.6, which is indicative of the existence of at least two binding sites in the enzyme for PEP, with some degree of interaction between them.

Effect of ADP concentration. Pyruvate kinase isoenzymes from *S. typhimurium* showed hyperbolic kinetics when the initial velocity was plotted against ADP concentrations at several fixed PEP concentrations. Double-reciprocal plots of the data gave a family of straight lines that intersected on the $1/v$ axis, giving estimated apparent K_m and $V_{max.}$ values of 0.21 mM and 74 ± 5.2 nmol of pyruvate formed/min for pyruvate kinase I and 0.16 mM and 35 ± 3.1 nmol of pyruvate formed/min for pyruvate kinase II.

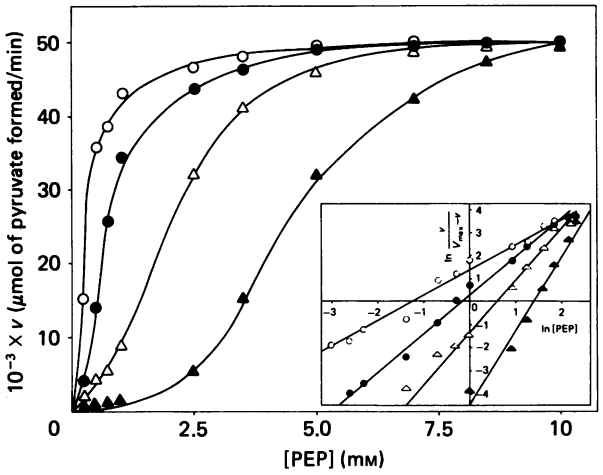

Fig. 3. Effect of fructose 1,6-bisphosphate on the kinetic response of pyruvate kinase I with respect to PEP concentration

Assays were carried out in 30 mM-Hepes buffer, pH 6.8, at 30 °C with 3 μg of purified pyruvate kinase I, 1.0 mM-ADP and 5 mM-$MgCl_2$. Fructose bisphosphate was added as indicated: ▲, none; △, 0.01 mM; ●, 0.05 mM; ○, 0.10 mM. Hill plots of the data are shown in the inset.

Effect of Mg^{2+} concentration. Pyruvate kinases showed sigmoidal kinetics when Mg^{2+} was the variable substrate, indicating positive co-operative interactions between the bivalent cation and the isoenzymes. This behaviour was obtained both for a saturating PEP concentration and fixed ADP concentrations, and for a saturating ADP concentration and fixed PEP concentrations (Table 3). Hill plots were linear, with slopes of 2.5 and 1.2 for pyruvate kinase I and II respectively. As the kinetic parameters remain practically unchanged, it is reasonable to think that the binding sites in both isoenzymes for ADP, PEP and Mg^{2+} should be independent. The rate of

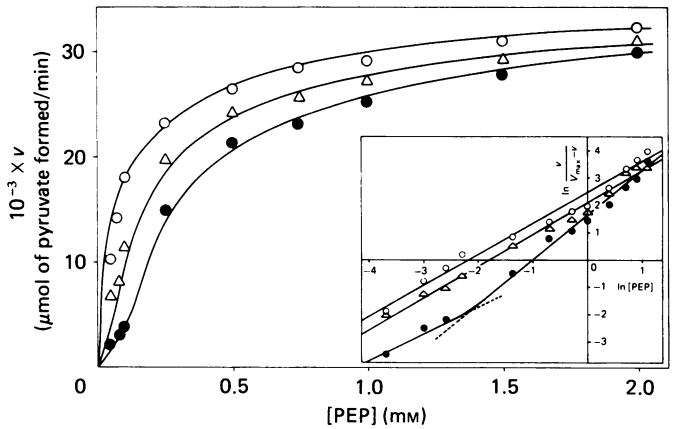

Fig. 4. Effect of AMP on the kinetic response of pyruvate kinase II with respect to PEP concentration

Assays were carried out in 30 mM-Hepes buffer, pH 6.8, at 30 °C, with 4 μg of purified pyruvate kinase II, 0.25 mM-ADP and 5 mM-$MgCl_2$. AMP was added as indicated: ●, none; △, 0.1 mM; ○, 1.0 mM. Hill plots of the data are shown in the inset.

catalysis reactions increased with Mg^{2+} concentrations to 3 mM for pyruvate kinase I and 5 mM for form II, but then diminished from 7 mM and 10 mM respectively.

Influence of some effector compounds on the pyruvate kinases

Effect of fructose 1,6-bisphosphate on pyruvate kinase I activity. Fructose 1,6-bisphosphate acts as a very sensitive modulator of the enzyme activity, by gradually decreasing the sigmoidicity of the kinetics toward PEP. The activation by fructose 1,6-bisphosphate has been reported in pyruvate kinases from different biological sources (Malcovati *et al.*, 1973; Ng & Hamilton, 1975; Flynn & Bowman, 1980; León *et al.*, 1982; Gaitan *et al.*, 1983; Busto *et al.*, 1985). The kinetic response to PEP concentration in the presence of fructose 1,6-bisphosphate was normalized to a hyperbolic form on a velocity-against-substrate-concentration plot as shown in Fig. 3. In Hill plots of the saturation data, the Hill coefficient decreases from 3.0 in its absence to 1.0 in the presence of 0.1 mM-fructose 1,6-bisphosphate. Likewise, the $s_{0.5}$ for PEP was also altered, decreasing from 4.5 mM in its absence to 0.21 mM in the presence of 0.1 mM-fructose 1,6-bisphosphate. The concentration of fructose 1,6-bisphosphate required for maximal activation varies with the PEP concentration: 0.05 mM-fructose 1,6-bisphosphate at 0.25 mM-PEP gives a 25-fold stimulation, whereas no effect is observed at 10 mM-PEP.

The effect of fructose 1,6-bisphosphate on the kinetic response for ADP was investigated, by maintaining 2.5 mM-PEP concentration and with three (0.01, 0.05 and 0.10 mM) fixed fructose 1,6-bisphosphate concentrations. Double-reciprocal plots of the saturation data gave a series of straight lines that intersected on the $1/v$ axis. The apparent $V_{max.}$ value was unchanged, but the affinity of the enzyme for ADP decreased when the fructose 1,6-bisphosphate concentration was increased. The effect of fructose 1,6-bisphosphate on pyruvate kinase I was studied at 1.0 mM-ADP concentration, four fixed PEP concentrations (0.5, 1.0, 2.5 and 5.0 mM) and variable fructose 1,6-bisphosphate concentration. The saturation curves for fructose 1,6-bisphosphate showed decreasing sigmoidicity of the kinetics when PEP concentration increased, the sigmoidal saturation curve becoming hyperbolic, while the Hill coefficient changed from 2.2 to 1.0.

Effect of AMP on pyruvate kinase II activity. The effect of AMP on the kinetics was studied at 5 mM-Mg^{2+}, 0.25 mM-ADP, three fixed AMP and varied PEP concentrations (Fig. 4). The experiments were repeated under the same conditions, except that the ADP concentration was 1.0 mM. In both cases Hill plots of the saturation data gave lines with a maximal slope (h) which decreased as the AMP concentration increased. When the experiments were carried out at four fixed AMP concentrations (0.05, 0.1, 0.5 and 1.0 mM), variable ADP concentrations and for 0.25 mM- and 1.0 mM-PEP, hyperbolic kinetic plots were obtained. Double-reciprocal plots gave a family of lines which intersected in a point on the $1/v$ axis. Therefore AMP does not seem to alter the $V_{max.}$ with respect to ADP, but decreases the affinity of the enzyme for this substrate. When variable AMP concentration was used, hyperbolic kinetics were obtained for both 0.25 mM-PEP and two fixed ADP concentrations (0.25 and 0.10 mM), as well as for 0.25 mM-ADP and two fixed PEP concentrations (0.10

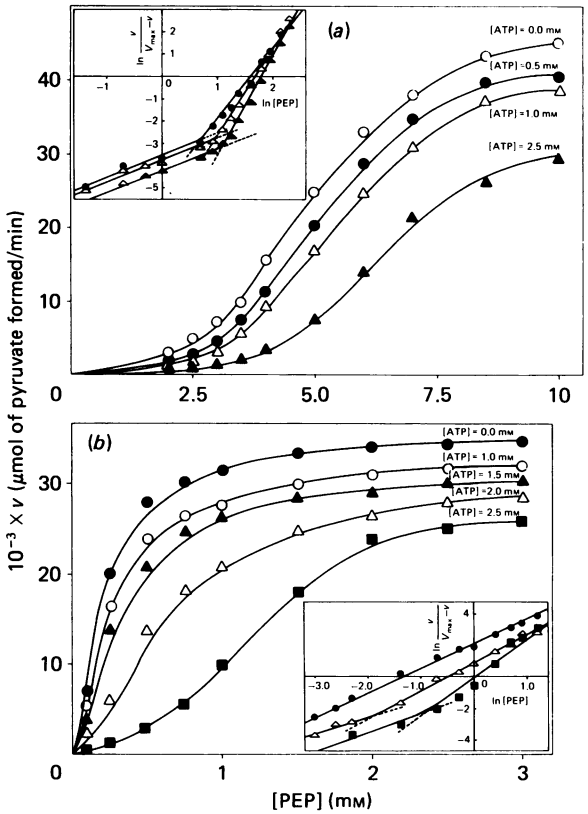

Fig. 5. Kinetics of pyruvate kinase from *S. typhimurium* in presence of ATP

The assay mixture, contained in 1 ml: 30 mM-Hepes buffer, pH 6.8, 5 mM-$MgCl_2$, 0.15 mM-NADH, 5 μg of crystalline lactate dehydrogenase, 1.0 mM-ADP, variable PEP concentration, the indicated ATP concentration and either 3 μg of purified pyruvate kinase I (*a*) or 4 μg of pyruvate kinase II (*b*). Hill plots of the data are shown in the insets.

Table 4. Specific activities of pyruvate kinases in extracts of *Salmonella typhimurium* grown on different carbon sources

The assay mixture contained, in 1 ml: 30 mM-Hepes buffer, pH 6.8, 5 mM-$MgCl_2$, 0.15 mM-NADH, 5 μg of crystalline lactate dehydrogenase and bacterial extract (approx. 40 μg of protein). After measurement of the endogenous rate of NADH oxidation, the reaction was initiated by the addition of 10 mM-PEP and the value obtained was considered as the sum of the pyruvate kinase I and II activities. The assay was repeated in the same conditions but with 1 mM-PEP, and the values obtained was considered to be 85% of the form II.

Carbon source	Specific activity (unit/mg of protein)	
	Form I	Form II
Glucose	0.34	0.28
Fructose	0.32	0.28
Galactose	0.27	0.25
Glycerol	0.29	0.27
PEP	0.14	0.22
Pyruvate	0.11	0.20
Succinate	0.11	0.23
Acetate	0.09	0.45

and 0.25 mM). Analysis of these kinetic patterns suggests that AMP is a linear competitive activator that affects only the affinity of the enzyme.

Effect of ATP on pyruvate kinase I and II activities. ATP, a product of the reaction, is an inhibitor of pyruvate kinases I and II. At 1.0 mM-ADP, three fixed ATP concentrations and variable PEP concentration, form I showed sigmoidal kinetics. As shown in Fig. 5(*a*), ATP is an inhibitor that alters the co-operativity of the PEP binding to the enzyme and increases the $s_{0.5}$ value from 4.7 to 6.3 mM. Likewise, the Hill coefficient increases from 3.0 without ATP to 4.3 in the presence of 2.5 mM-ATP. Pyruvate kinase I showed hyperbolic saturation curves for a high PEP concentration (10 mM), three fixed ATP concentrations (0.5, 1.0 and 2.5 mM) and variable ADP concentration. Double-reciprocal plots of the saturation data gave a family of lines that intersected on the $1/v$ axis. From plots of initial rates versus ATP concentration, at three fixed ADP concentrations (0.2, 0.5 and 0.85 mM), it is easy to deduce that the inhibitor can form Enzyme–ATP and Enzyme–ATP–ADP complexes, where the latter is capable of giving products and having partially competitive inhibition (Segel, 1975).

Pyruvate kinase II showed sigmoidal kinetics at 1.0 mM-ADP, four fixed ATP concentrations and variable PEP concentration. The $s_{0.5}$ value for PEP increased from 0.2 mM without ATP to 1.2 mM in the presence of 2.5 mM-ATP, whereas the $V_{max.}$ was constant. From a Hill plot of the saturation data, as illustrated in Fig. 5(*b*), the Hill coefficient increases from 1.6 to 2.6. When pyruvate kinase II activity was assayed at high PEP concentration (3.0 mM), five fixed ATP concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mM) and variable ADP concentration, hyperbolic curves were obtained. Double-reciprocal plots of the saturation data for the five different ATP concentrations gave a series of straight lines which intersected in a point on the $1/v$ axis. From a Dixon-like plot of $1/v$ versus ATP concentration, it can be deduced that ATP is a competitive inhibitor, with a K_i value of 0.57 mM.

Effect of carbon source on the pyruvate kinase I and II activities. Taking into account the different behaviour of pyruvate kinase I and II with PEP concentration, it is possible to devise a method for measuring the activity of the two forms in extracts containing both of them, similar to that described by Malcovati *et al.* (1973). So, in the extracts of cells grown on different carbon sources and assayed at a saturating concentration of PEP (10 mM), the activities of the pyruvate kinases were at 100%. Then the extracts were assayed at 1.0 mM-PEP, at which form I is almost inactive and form II is about 85% active. The results reported in Table 4 show that the activity of pyruvate kinase I varies with the nature of the growth substrate, whereas that of form II appears to be independent of the growth conditions. That is, form I contributes to a greater extent to the total activity than does form II when cells are grown on glycolytic substrates, and in these conditions the ratio of specific activities was two times greater than in extracts of cells grown on gluconeogenic substrates.

DISCUSSION

In order better to understand the physiological reasons for the occurrence of these two pyruvate kinase isoenzymes in *S. typhimurium*, they were purified and their properties studied. Some properties of pyruvate kinase I and II appear to be similar: both enzymes were absolutely dependent on bivalent cations, their activities being higher in Hepes buffer than in other buffers, and they showed sigmoidal kinetics with PEP and Mg^{2+} when these compounds were variable substrates, but hyperbolic kinetics with ADP. However, they differ in M_r values, in amino acid composition and in heat-stabilities; likewise, significant differences exist between the kinetic parameters of the two isoenzymes and in the compounds that activate the enzymes. From these observations it seems reasonable to conclude that the two forms are, rather than interconvertible enzymes, different proteins. Also, some differences can be seen between pyruvate kinase from *S. typhimurium* and the homologous enzymes from *E. coli*, with respect to optimum pH, to $s_{0.5}$ values and to Hill coefficients, when PEP was the variable substrate. Furthermore, the pyruvate kinases from *E. coli* exhibit sigmoidal curves for ADP in the presence of non-saturating concentrations of PEP (Waywood & Sanwal, 1974; Waywood *et al.*, 1975; Kotlarz *et al.*, 1975), whereas the isoenzymes reported here show similar behaviour to that of pyruvate kinases from different biological sources (Flynn & Bowman, 1980; León *et al.*, 1982; Busto *et al.*, 1985), where sigmoidal rate–concentration curves are given only with PEP.

The isoenzymes from *S. typhimurium* require the presence of a bivalent cation, such as Mg^{2+}; and the concentration required for maximum activity is dependent on the concentrations of ADP and PEP used. Maxima in the relationship of the enzyme activity to bivalent-cation concentration have been observed with other kinases (Hunsley & Suelter, 1969; Ng & Hamilton, 1975; Morris *et al.*, 1983; Herranz & Ruiz-Amil, 1985).

Ainsworth & Mcfarlane (1975) suggested that the maxima arise because the true substrates of the enzyme are free PEP, ADP and Mg^{2+}. In *S. typhimurium* this hypothesis is supported by the fact that the binding sites in the pyruvate kinases for ADP, PEP and Mg^{2+} seem to be independent. Thus the rate of the catalysed reaction increases with Mg^{2+} concentration because Mg^{2+} acts as a substrate, but diminishes with greater Mg^{2+} concentrations because PEP and ADP are progressively removed as their metal-bound complexes.

A characteristic of pyruvate kinase I is the heterotropic activation by fructose 1,6-bisphosphate, which interacts with the enzyme, eliciting marked conformational changes which conduce to abolish the sigmoidal response and increase the affinity in the binding of PEP to the enzyme. The activation by fructose 1,6-bisphosphate appears to affect the PEP binding in particular, since the kinetic response with ADP is not altered. This activation is synergistic between PEP and fructose 1,6-bisphosphate, because at a high concentration of PEP no heterotropic activation is observed, and likewise at a high fructose 1,6-bisphosphate concentration no homotropic interaction between PEP sites is found. No change in the $V_{max.}$ value is observed in the presence of fructose 1,6-bisphosphate, and this enzyme may be classified as a 'pure K' system (Monod *et al.*, 1965). Similar conjunction between fructose 1,6-bisphosphate and PEP has been described for pyruvate kinase from *Trypanosoma brucei* (Flynn & Bowman, 1981) and *Phycomyces blakesleeanus* (Busto *et al.*, 1985).

Pyruvate kinase II is activated by AMP, which is a property of some homologous enzymes from bacterial sources, such as those from *E. coli* (Waywood *et al.*, 1975; Valentini *et al.*, 1979), *Brevibacterium flavum* (Ozaki & Shiio, 1969) and *Bacillus licheniformis* (Touminen & Bernlohr, 1971).

ATP appears to be an effective inhibitor for both pyruvate kinase from *P. blakesleeanus* (Busto *et al.*, marked decrease in the Hill coefficient *h*. Except for pyruvate kinase from *P. blakesleeanus* (Bustos *et al.*, 1985), where ATP does not affect $s_{0.5}$ or *h*, the behaviour of pyruvate kinase from *S. typhimurium* was similar to that described for prokaryotic and eukaryotic cells (Maeba & Sanwal, 1968; Benziman, 1969; Waywood *et al.*, 1975; Ng & Hamilton, 1975; Behm & Bryant, 1980; Flynn & Bowman, 1980; Gaitan *et al.*, 1983).

On the other hand, the operation of the hexose phosphotransferase system (PTS), for the uptake and phosphorylation of certain monosaccharides, which should be an alternative mechanism to convert PEP into pyruvate, does not seem to have any influence on the pyruvate kinase activities, because the values obtained in cells grown on glucose and fructose (Table 4), which are transported by the PTS system, were similar to those for cells grown on galactose and glycerol, which are transported into the bacteria by other systems. The importance of the possible reaction sequence involving PEP carboxylase, malate dehydrogenase and malic enzyme and the action of a phosphatase (Alonso *et al.*, 1979) in the formation of pyruvate from PEP is difficult to evaluate in relation to pyruvate kinase activity; the latter enzyme seems different from the periplasmic phosphatases described by Kier *et al.* (1975), since *S. typhimurium* LT-2 has an appropriate uptake system for PEP (Saier *et al.*, 1975), and therefore its role in the cell remains unknown.

The kinetic properties of the pyruvate kinases deserve to be discussed in some detail according to their physiological significance. Taking into account that the intracellular concentration of fructose 1,6-bisphosphate is relatively high when cells grow on glucose, fructose and glycerol (Lowry *et al.*, 1971), it may be correct to think that form I acts mainly in glycolytic conditions. In gluconeogenic conditions, the concentrations of fructose 1,6-bisphosphate are much lower that in glycolytic conditions, and the activity of the enzyme should be drastically decreased; if a value of 0.24–0.30 mM for the intracellular PEP concentration during growth on gluconeogenic compounds can be assumed to be correct (Zwaig *et al.*, 1970; Lowry *et al.*, 1971), it would seem reasonable to suppose that form I is inactive, whereas form II can catalyse the reaction at 30–43% of the maximum reaction rate. Because of the less marked sigmoidicity of form I towards PEP, its $s_{0.5}$ is of the same order as the average concentration of the substrate during growth on gluconeogenic compounds, and its activity might then be modulated by fluctuation of the PEP concentration. On the other hand, AMP was found to be an effective activator, and its activation of the enzyme activity might be very important because an increase in its concentration is a chemical signal of a decrease in the energy charge of the cells, and its activation could provide a rapid transformation of PEP into ATP.

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