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Kinetic Properties of Rat Liver Pyruvate Kinase at Cellular Concentrations of Enzyme, Substrates and Modifiers

By WAYNE FLORY,* BENIGNO D. PECZON, ROGER E. KOEPPE
and
H. OLIN SPIVEY

Department of Biochemistry, Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma 74074, *U.S.A*

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Kinetic properties of rat liver pyruvate kinase type I at pH7.5 and 6.5 were studied with physiological ranges of substrates, modifiers and Mg^{2+} concentrations at increasing enzyme concentrations, including the estimated cellular concentrations (approx. 0.1mg/ml). Enzyme properties appear unaffected by increased enzyme concentration if phosphoenolpyruvate, fructose 1,6-diphosphate and inhibitors are incubated with enzyme before starting the reaction with ADP. Our data suggest that minimum cellular concentrations of MgATP and L-alanine provide virtually complete inhibition of pyruvate kinase I at pH7.5. The most likely cellular control of existing pyruvate kinase I results from the strong restoration of enzyme activity by the small physiological amounts of fructose 1,6-diphosphate. Decreasing the pH to 6.5 also restores pyruvate kinase activity, but to only about one-third of its activity in the presence of fructose 1,6-diphosphate. Neither pyruvate nor 2-phosphoglycerate at cellular concentrations inhibit the enzyme significantly.

Studies of rat liver pyruvate kinase I (EC 2.7.1.40) previously termed type L, indicate that it may have a significant regulatory role in glycolysis (Rozengurt *et al.*, 1969; for other pertinent references see introductory paragraph of Flory & Koeppe, 1973). It is difficult, however, to evaluate the probable physiological activity of the enzyme, or factors most important in its regulation from previously available information. Few investigators use phosphate in their buffers, and the relationship between Mg^{2+} and adenine nucleotide concentrations is sometimes ignored. Often the concentrations of fructose 1,6-diphosphate and phosphoenolpyruvate are unphysiological, as are the ATP/ADP ratios, and reproducibility of enzyme preparations, particularly with respect to fructose 1,6-diphosphate activation of crude enzymic preparations, is sometimes unsatisfactory. Tests for inhibitions by pyruvate or 2-phosphoglycerate have not been published to our knowledge.

Previous kinetic measurements with pyruvate kinase I have been limited to enzyme concentrations below approximately 1μg/ml, although estimates of the intracellular concentration of type I enzyme indicate that it may attain concentrations of 200μg/g of rat liver [from Tables IV and V of Tanaka *et al.* (1967)]. Several examples exist where increased enzyme concentrations cause changes in their intrinsic kinetic or binding properties (e.g. Hofer, 1971; Frieden, 1970).

The present paper reports results of studies to assess the simultaneous effects of H^+, fructose 1,6-diphosphate, phosphoenolpyruvate, L-alanine, Mg^{2+}, adenine nucleotides and enzyme concentrations at physiological concentrations on enzymic activity in phosphate buffer. The enzyme preparations used exhibited reproducible fructose 1,6-diphosphate stimulation unaffected by temperature, as reported by Flory & Koeppe (1973).

Experimental

Materials

ATP (Sigma grade), ADP (grade I), NAD^+ (grade III), potassium phosphoenolpyruvate, fructose 1,6-diphosphate tetrasodium salt (Sigma grade), bovine heart lactate dehydrogenase (type III) suspension in $(NH_4)_2SO_4$, 2-mercaptoethanol, cellulose (medium) and DEAE-Sephadex A-50 were from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-Alanine and dithiothreitol were from Calbiochem, Los Angeles, Calif., U.S.A.; EDTA (disodium salt), $(NH_4)_2SO_4$ (enzyme grade) and Tris base (ultra pure) were from Schwarz–Mann, Orangeburg, N.Y., U.S.A., rabbit muscle phosphofructokinase suspen-

* Present address: Biochemistry Department, School of Medicine, University of South Dakota, Vermillion, South Dakota 57069.

sion in 2M-$(NH_4)_2SO_4$ was from Boehringer Mannheim, New York, N.Y., U.S.A., and polyethylene glycol (15000–20000 average mol.wt.) was from MC/B Manufacturing Biochemists, Norwood, Ohio, U.S.A.

Methods

Enzyme assays. The standard conditions used for the pyruvate kinase assays were 0.6mM-phosphoenolpyruvate, 1.5mM-MgADP, 1.0mM-fructose 1,6-diphosphate, 0.14mM-NADH, approx. 10 units of bovine heart lactate dehydrogenase/ml, and 8.0mM-$MgSO_4$ in 5.0mM-potassium phosphate buffer at pH7.5 containing 150mM-KCl and 5mM-2-mercaptoethanol. Assays with approximately 0.1 μg of enzyme/ml were made at 340nm in a 1 cm-path-length cuvette at 25°C in a Coleman 124 spectrophotometer equipped with a Coleman 165 recorder. This assay at 25°C gave the same reaction rates within experimental error as that described by Tanaka *et al.* (1967), but avoided the inconvenience of using 37°C. The essentially twofold greater enzymic activity at 25°C in our assay solution compared with Tanaka's at 37°C is due principally to the presence of 1.0mM-fructose 1,6-diphosphate in our assay, which is also recommended in order to avoid possible errors from residual fructose 1,6-diphosphate which frequently occurs in crude pyruvate kinase preparations (Flory & Koeppe, 1973). For all stopped-flow measurements, the lactate dehydrogenase was dialysed exhaustively against the assay buffer before use and its concentration (units of enzyme/ml) determined by assay at 340nm with 0.4mM-pyruvate and 0.15mM-NADH in the same buffer. Potential contaminating enzymes (see below) were assayed by methods suggested in the catalogue of Boehringer Mannheim Corp., except for fructose 1,6-diphosphatase, which was assayed by the method of Pogell *et al.* (1968), but at pH values of 7.5 and 6.5 rather than at the pH optimum of the diphosphatase.

Preparation of enzyme. Data in Table 1 were obtained on Sephadex-treated crude enzyme, prepared as described by Flory & Koeppe (1973). All other data were obtained with pyruvate kinase I, purified by the method of Tanaka *et al.* (1967) up to the DEAE-Sephadex A-50 chromatography. This differed by the use of a linear gradient between equal volumes (250ml) of 0.1 and 0.2M-KCl in the 5mM-Tris buffer for elution of the enzyme, and the pooling of all fractions with greater than 3 units of enzyme/ml. This use of a larger fraction of the peak than retained by Tanaka *et al.* (1967) was due to the large quantities of enzyme needed for our experiments. The pooled enzyme, stabilized by dialysis against 0.02M-acetate buffer at pH5.0 containing 0.2mM-ADP, 0.2mM-phosphoenolpyruvate and 1mM-dithiothreitol, was loaded on a cellulose–phosphate column and eluted as described by Tanaka *et al.* (1967). Fractions with appreciable activity were pooled and the enzyme concentration was increased by dialysis against polyethylene glycol. The enzyme was then dialysed against the buffers used in stopped-flow experiments at pH7.5 or 6.5. These buffers were 10mM-phosphate, 8mM-$MgSO_4$, 5mM-2-mercaptoethanol and sufficient KCl (~135–145mM) to give 159mM-K^+ after pH adjustment of KH_2PO_4 with KOH. In one enzyme purification used in stopped-flow measurements fractional precipitation by polyethylene glycol, instead of cellulose–phosphate chromatography, resulted in lower contamination with fructose 1,6-diphosphatase. The enzyme solutions were brought to 5, 10, and 15% saturation with polyethylene glycol by addition of a 50% saturated stock solution. Precipitates were separated by 30min centrifugation at 100000*g* at each of these fractional precipitations, and the pellet from the 15% saturated solution was dissolved in the 10mM-phosphate buffer, pH7.5.

A purity of 65% for pyruvate kinase I from the cellulose–phosphate column was calculated from its specific activity relative to that of the crystalline preparation of Tanaka *et al.* (1967). Protein concentrations were measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. Activities of myokinase, aldolase and L-alanine transaminase were not detected in this preparation. Phosphatase activity against fructose 1,6-diphosphate was 0.08% of the pyruvate kinase activity at pH6.5. This contaminating activity was extremely hard to decrease further. Since our conclusions are unaffected by its presence at this concentration, further purification was not sought.

Stopped-flow measurements. A Gibson–Durrum stopped-flow spectrophotometer with a 0.234cm-path-length cell was used at 340nm and 25°C for measurements above 0.5 μg of enzyme/ml. Instrument calibrations and methods are described by Peczon & Spivey (1972). Activities measured on 0.1 μg of enzyme/ml in both stopped-flow and Coleman 124 spectrophotometers were identical within experimental errors (~10%). A minimum of two and often more replicate kinetic measurements were made for each condition, and all experiments were repeated with at least two separate purifications of enzyme. All conditions tested in the stopped-flow experiments at high enzyme concentrations were also examined with lower enzyme concentrations in the Coleman 124 within 1 h of the corresponding stopped-flow measurements. Samples of enzyme taken from the syringes before and after stopped-flow experiments verified enzyme stability during experiments.

Results and Discussion

This discussion will assume ranges of physiological concentrations reported in the following references:

1. Williamson (1966); 2. Williamson *et al.* (1966*a*); 3. Williamson *et al.* (1966*b*); 4. Exton & Park (1969); 5. Ross *et al.* (1967); 6. Ballard (1971); 7. Start & Newsholme (1968); 8. Burns *et al.* (1970); 9. Long (1961*a*,*b*,*c*) 10. Garber & Hanson (1971). These concentrations (μmol/g wet wt.) with the above reference numbers in parenthesis are: ADP, 0.4–1.5 (1–7); phosphoenolpyruvate, 0.02–0.5 (1–5); ATP, 1–3 (1–7); fructose 1,6-diphosphate, 0.002–0.03 (1–5): L-alanine, 0.5–3 (8); pyruvate, 0.01–0.4 (1–4); 2-phosphoglycerate 0.01–0.15 (1, 2 and 4); K^+, 100 (9); Mg^{2+}, 5–10 (9); Cl^-, 28 (9); phosphate, 4–10 (9 and 10). Pyruvate kinase I concentrations range from 0.05mg/g of liver on a high-protein diet to 0.2mg/g of liver with a high-carbohydrate diet (Tanaka *et al.*, 1967). Species in our assay solutions at unphysiological concentrations [Cl^- and 2-mercaptoethanol, and with crude enzyme, $(NH_4)_2SO_4$ and 50mM-P_i], were shown to have negligible effect on pryuvate kinase activities at the concentrations used.

Possible subcellular compartmentation and binding of the above components make it difficult to estimate their effective concentrations *in vivo*. Enzyme concentrations calculated on the basis of whole tissue will underestimate local concentrations, though this error may not be significant with pyruvate kinase, since it is a cytoplasmic enzyme. In any case, useful insights can be gained by studying a range of concentrations around estimates of the cellular values, especially if these, and other complexities of the cellular environment are considered in interpreting the data.

Results with low concentrations of enzyme

Table 1 shows that in crude enzyme preparations at pH7.5, liver pyruvate kinase (I and II) activity in the presence of low cellular concentrations of MgATP and L-alanine was only 5% of its maximum value. Data at pH7.5 and 6.5 from the more purified enzyme differ significantly from those in Table 1 only in the lower activity with low concentrations of fructose 1,6-diphosphate [0.16% of maximum value, with no fructose 1,6-diphosphate, see Table 1 of Spivey *et al.* (1974)] owing to the lack of pyruvate kinase II. Therefore for results with low concentrations of enzyme the more extensive data with Sephadex-treated crude enzyme are cited. These results were completely reproducible and unaffected by incubation at 0° or 25°C if fructose 1,6-diphosphate was adequately removed from the enzyme preparation (Flory & Koeppe, 1973). Addition of 0.02mM or more fructose 1,6-diphosphate to low concentrations of enzyme (less than 1μg/ml) completely reversed inhibitions by MgATP and L-alanine giving approx. 50% of the maximum activities, the same value

Table 1. *Effects of fructose 1,6-diphosphate and pH on inhibited rat liver pyruvate kinase at low concentrations of enzyme*

Measurements were made (with the Coleman spectrophotometer) at 25°C with Sephadex-treated crude enzyme fractions in 50mM-potassium phosphate buffer at the indicated pH value containing: 1.5mM-MgADP, 0.15mM-phosphoenolpyruvate, 1.75mM-MgATP, 0.5mM-L-alanine, the indicated quantities of fructose 1,6-diphosphate, 3mM-$MgSO_4$, about 0.05 unit of pyruvate kinase/ml, 25 units of lactate dehydrogenase/ml, 0.14mM-NADH, 30mM-$(NH_4)_2SO_4$, 1mM-2-mercaptoethanol. Results on more purified enzyme differ significantly only in having much less activity with low concentrations of fructose 1,6-diphosphate (0.16% of maximum value, with no fructose 1,6-diphosphate), owing to lack of pyruvate kinase II. Percentage activity in the standard assay is specified in the Experimental section, where specific activity = 388 units/mg.

		Activity (%)				
	[Fructose 1,6-diphosphate]					
pH	(μM) ...	0	5	10	20	50
7.5		5.0	13	30	43	48
7.0		8.0	37	50	57	58
6.5		32	57	62	63	62

obtained in the absence of inhibitors with 0.15mM-phosphoenolpyruvate ($\simeq K_m$ value). Increased H^+ concentration (pH6.5) in the absence of fructose 1,6-diphosphate can substantially, but not completely decrease both MgATP and L-alanine inhibition of liver pyruvate kinase. Only 5μM-fructose 1,6-diphosphate was required (Table 1) to overcome these inhibitions at pH6.5, for example.

Extensive measurements (results not presented) also demonstrated the following properties of the liver enzyme. Significant inhibition by 0.5–1.5mM-MgADP, with or without MgATP and L-alanine was not observed, in contrast with the report by Tanaka *et al.* (1967), but in agreement with results of Carminatti *et al.* (1968). Variation of MgADP in the range 0.5–1.5mM had little effect on enzymic activity. An excess above nucleotide concentrations of 3–8mM-Mg^{2+} provides near optimum conditions in agreement with results of Carminatti *et al.* (1968). Phosphoenolpyruvate (0.075–0.6mM) had little effect on pyruvate kinase activity in the presence of the inhibitors and absence of fructose 1,6-diphosphate at pH7.5. Addition of 5μM-fructose 1,6-diphosphate or lowering the pH to 6.5, however, provides substantial control of enzymic activity by phosphoenolpyruvate; at pH6.5 with no fructose 1,6-diphosphate, an increase of phosphoenolpyruvate from 0.075 to 0.6mM caused an increase in enzymic activity

from 10 to 56% of maximum values, for example. Simultaneously increasing MgATP and L-alanine concentrations to 3.5 and 2.0mM respectively, and decreasing MgADP to 0.5mM, generally increases the amount of inhibition by two- to three-fold. Inhibitions of enzyme by 0.6–4.8mM-pyruvate were negligible at pH6.8 or 7.2 and with 0.3–1.2mM-phosphoenolpyruvate; inhibition by 1.2mM-2-phosphoglycerate was also negligible at pH7.5 and with 0.3–1.2mM-phosphoenolpyruvate, and less than 50% inhibitions were observed at 4.8mM-2-phosphoglycerate.

Results with high concentrations of enzyme

Substrate and modifier concentrations used for the data in Table 1 were chosen for measurements with higher enzyme concentrations at pH7.5 and 6.5. Some changes in KCl, Mg^{2+}, P_i and thiol concentrations were adopted for these and subsequent experiments after demonstrating that the enzyme's response to fructose 1,6-diphosphate was essentially unaffected by these changes, although the absolute activity of enzyme was slightly higher in the new buffer. At pH7.5, unless fructose 1,6-diphosphate was incubated with high concentrations of enzyme before starting the catalytic reaction, more than 0.1mM-fructose 1,6-diphosphate was required to completely overcome inhibition by MgATP and L-alanine (Table 2), whereas 0.02mM-fructose 1,6-diphosphate was adequate if it was incubated with the enzyme. This necessity for incubation is discussed by Spivey *et al.* (1974) and results from the slowness in the activation of the inhibited enzyme by fructose 1,6-diphosphate, relative to the rate of the catalytic reaction at high concentrations of enzyme.

Table 3 shows the effectiveness of decreased pH in overcoming enzymic inhibitions as a function of enzyme concentration. Although a small decrease in enzyme activity with increasing enzyme concentration appears, we believe this to be the consequence of a slow activation by phosphoenolpyruvate rather than a change in the intrinsic properties of the enzyme with increasing concentration. Lag transients in reaction progress curves after addition of phosphoenolpyruvate to the enzyme were also observed, which are in keeping with this view as explained above. Phosphoenolpyruvate was not incubated with enzyme in these measurements since previous experiments revealed significant conversion of phosphoenolpyruvate into pyruvate occurred under these circumstances, owing

Table 2. *Fructose* 1,6-*diphosphate activation of inhibited pyruvate kinase I at pH* 7.5

Fructose 1,6-diphosphate was incubated with enzyme before the catalytic reaction (values in parentheses), or rapidly mixed with the enzyme at the start of the catalytic reaction. In the latter case, activities were calculated from the linear portions of the progress curves following the initial transients (see Fig. 1 and the text of Spivey *et al.*, 1974). Concentrations after mixing of equal volumes from stopped-flow drive syringes were 20 units of bovine heart lactate dehydrogenase/unit of pyruvate kinase I; 0.15mM-phosphoenolpyruvate; 1.5mM-MgADP; 1.75mM-MgATP; 0.5mM-L-alanine; 0.5mM-NADH, and the indicated quantities of fructose 1,6-diphosphate and enzyme in 10mM-potassium phosphate buffer, pH 7.5, containing 140mM-KCl, 8mM-$MgSO_4$ and 5mM-2-mercaptoethanol. The enzymes were stored in drive syringe 1; MgADP, phosphoenolpyruvate and NADH were stored in syringe 2. Fructose 1,6-diphosphate and all other components were distributed equally between the drive syringes when fructose 1,6-diphosphate was incubated with enzyme. Otherwise, fructose 1,6-diphosphate was stored in drive syringe 2, only. Solutions were maintained at 25°C. Percentage activity in the standard assay is specified in the Experimental section, where specific activity of the pure enzyme is 388 units/mg.

Enzyme activity (units/ml)	[Fructose 1,6-diphosphate] (mM) ...	Activity (%)					
		0	0.02	0.05	0.1	1.0	2.0
20		0.15	10	29	42	53	55
6.2			19 (57)				
0.07			49 (54)				

Table 3. *Activity of pyruvate kinase type I at pH* 6.5 *as a function of fructose* 1,6-*diphosphate and enzyme concentration*

For assay conditions, except for pH change, see the legend to Table 2. When used, fructose 1,6-diphosphate was in both syringes. Percentage of activity in the standard assay is specified in the Experimental section.

Fructose 1,6-diphosphate concn. (μM)	[Enzyme] (μg/ml) ...	Activity (%)				
		0.13	0.8	8.6	13	98
0		18	17	—	13	11
20		53	—	38	—	—

most likely to contamination of ATP by ADP. However, longer transient times for full inhibition of enzyme by MgATP required its incubation with enzyme. Further purification of our ATP was not attempted since changes in enzyme activity with enzyme concentration were so slight and explicable in these data.

We conclude that in the presence of physiological concentrations of substrates and known modifiers, liver pyruvate kinase I activity may be regulated at pH7.5 from less than 1% to 50% of its maximum activity, principally by fructose 1,6-diphosphate. Decreased pH values (6.5) provided substantial, but less activation except in combination with high concentrations of phosphoenolpyruvate or low concentrations of fructose 1,6-diphosphate, where 50–60% of maximum activities were also obtained. Scrutton & Utter (1968) have summarized rates of glycolysis and gluconeogenesis for rat liver as 0.2 and 1.7–1.8μmol/min per g wet wt. at 37°C respectively; the latter rates corresponding to liver perfused with pyruvate as precursor. Maximum pyruvate kinase activity of rat liver is given as 50μmol/min per g wet wt. at 37°C. Our results therefore strengthen the viewpoint that liver pyruvate kinase I is an important regulatory enzyme in concert with phosphofructokinase, since pyruvate kinase activity in liver with high concentrations of ATP and low concentrations of fructose 1,6-diphosphate may approach rates of glycolysis, especially with high-carbohydrate diets.

Unlike phosphofructokinase (Hofer, 1971), the properties of pyruvate kinase I appear independent of enzyme concentrations.

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References

Ballard, F. J. (1971) *Biochem. J.* **121**, 169–178
Burns, L. C., Meghal, S. K. & Koeppe, R. E. (1970) *Life Sci.* **9**, 1045–1051
Carminatti, H., Jimenez de Asua, L., Recondo, E., Passeron, S. & Rozengurt, E. (1968) *J. Biol. Chem.* **243**, 3051–3056
Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 1424–1433
Flory, W. & Koeppe, R. E. (1973) *Biochem. J.* **133**, 391–394
Frieden, C. (1970) *J. Biol. Chem.* **245**, 5788–5799
Garber, A. J. & Hanson, R. W. (1971) *J. Biol. Chem.* **246**, 589–598
Hofer, H. W. (1971) *Hoppe-Seylers Z. Physiol. Chem.* **352**, 998–1004
Long, C. (ed.) (1961*a*) *Biochemists' Handbook*, p. 677, D. Van Nostrand Co. Inc., New York
Long, C. (ed.) (1961*b*) *Biochemists' Handbook* p. 681, D. Van Nostrand Co. Inc., New York
Long, C. (ed.) (1961*c*) *Biochemists' Handbook* p. 780, D. Van Nostrand Co. Inc., New York
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Peczon, B. D. & Spivey, H. O. (1972) *Biochemistry* **11**, 2209–2217
Pogell, B. M., Tanaka, A. & Siddons, R. C. (1968) *J. Biol. Chem.* **243**, 1356–1367
Ross, B. D., Hems, R. & Krebs, H. A. (1967) *Biochem. J.* **102**, 942–951
Rozengurt, E., Jimenez de Asua, L. & Carminatti, H. (1969) *J. Biol. Chem.* **244**, 3142–3147
Scrutton, M. C. & Utter, M. F. (1968) *Annu. Rev. Biochem.* **37**, 249–302
Spivey, H. O., Flory, W., Peczon, B. D., Chandler, J. P. & Koeppe, R. E. (1974) *Biochem. J.* **141**, 119–125
Start, C. & Newsholme, E. A. (1968) *Biochem. J.* **107**, 411–415
Tanaka, T., Harano, Y., Sue, F. & Morimura, H. (1967) *J. Biochem. (Tokyo)* **62**, 71–91
Williamson, J. R. (1966) *Biochem. J.* **101**, 11C–14C
Williamson, J. R., Kreisberg, R. A. & Felts, P. A. (1966*a*) *Proc. Nat. Acad. Sci. U.S.* **56**, 246–254
Williamson, J. R., Wright, P. H., Malaisse, W. J. & Ashmore, J. (1966*b*) *Biochem. Biophys. Res. Commun.* **24**, 765–770