Mechanism of 'L'-Type Pyruvate Kinase from Rabbit Liver

EVIDENCE AGAINST PHOSPHOENZYME FORMATION

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The 'L'-type pyruvate kinase from rabbit liver does not catalyse exchange between phosphoenol[1-14C]pyruvate and pyruvate at either pH 8.5 or 6.2. Spectrophotometric experiments at pH 8.5 and 6.2 and gel-filtration experiments with [32P]phosphoenolpyruvate at pH 8.5 also fail to demonstrate phosphoenzyme formation. It is concluded that it is very unlikely that the enzyme has a phosphoenzyme mechanism.

It has been proposed that the 'L'-type isoenzyme of pyruvate kinase (EC 2.7.1.40) undergoes phosphorylation during the transfer of a phosphate group from phosphoenolpyruvate to ADP, since it exhibits steady-state kinetics typical of a Ping Pong mechanism at pH 6.2 (Macfarlane & Ainsworth, 1974). We report our attempts to demonstrate the existence of the phosphoenzyme at this pH and at pH 8.5. The latter pH was used since the present work was carried out as a preliminary to radioisotopic studies, which are most conveniently carried out at an alkaline pH.

Materials and Methods

All enzymes and substrates were from Boehringer Corp. (London) Ltd. (London W.5, U.K.). Scintillation chemicals were from Packard Instruments (Wembley, Middx., U.K.) and from Rohm and Hass (U.K.) Ltd. (Croydon, Surrey, U.K.). Radiochemicals were from The Radiochemical Centre (Amersham, Bucks., U.K.). Other chemicals were Reagent Grade or A.R. and were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

[32P]Phosphoenolpyruvate was prepared from [32P]P, and glyceraldehyde 3-phosphate by using 3-phosphoglycerate dehydrogenase, 3-phosphoglycerate kinase and pyruvate kinase (Dann & Britton, 1977). Sensitivity to hydrolysis by HgCl2, and enzymic conversion into ATP by pyruvate kinase showed over 98% of the label in phosphoenolpyruvate and less than 2% in Pi (Dann & Britton, 1977). [32P]Phosphoenolpyruvate was measured as total radioactivity in 0.5 ml samples added to a mixture of 0.5 ml of water and 10 ml of scintillation mixture [666 ml of toluene, 333 ml of Triton X-100, 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyl-oxazol-2-yl)benzene] by counting with a Packard model 3000 Series Tri-Carb liquid-scintillation counter. [32P]Pi was precipitated by the method of Sugino & Miyoshi (1964), dissolved in 300 μl of 1M-NaOH and transferred, with two washes (500 μl) of water, to vials and counted for radioactivity as above. 14C-labelled compounds were separated on polyethyleneimine-cellulose thin-layer plates (Anderman, East Molesey, Surrey, U.K.) by using a solvent of 0.3 m-ammonium formate adjusted to pH 3.0 with formic acid. Peaks were detected on a Packard model 7201 radiochromatogram scanner. Areas containing the radioactivity were cut out of the plates, placed in vials, and scintillation mixture containing 4% (w/v) Cab-O-Sil (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was added.

The dissociation constants for the Mg2+ complexes of phosphoenolpyruvate and ATP and ADP were taken from Wold & Ballou (1957) and Phillips et al. (1966) respectively. An equilibrium constant of 2125 for the pyruvate kinase reaction at pH 8.5, free 5 mM-Mg2+ and I 0.2 was assumed (Dann & Britton, 1977).

Purification of the enzyme

The enzyme was purified from rabbit liver by using a modification of the method of Irving & Williams (1973). After the heat step described by Irving & Williams (1973), the precipitated enzyme was dissolved in 20 mM-potassium phosphate buffer, pH 6.8, containing 30% (v/v) glycerol and 1 mM-mercaptoethanol. In this buffer the enzyme retained over 95% activity during overnight dialysis at 4°C to remove (NH4)2SO4 before chromatography at 4°C on a column (0.8 cm × 30 cm) of DEAE-cellulose. The enzyme was eluted from the column with 200 ml of a linear continuous KCl gradient (0–0.3 M-C1- ) in the buffer mixture described above. Fractions (3 ml) were collected, and those containing pyruvate kinase activity were pooled and (NH4)2SO4 was added to precipitate the enzyme (approx. 45% w/v). The purified enzyme was stored either in 45% (w/v) (NH4)2SO4 or in 50% (w/v) glycerol, and under these conditions it showed little loss of activity over a period of several weeks. Different batches of the enzyme had specific
The polyethyleneimine-cellulose (unpublished work) and phosphate, phosphoenolpyruvate, 0.1 M-MgCl₂ give I to 5mM-MgCl₂ gave activities of preparations. Enzyme activity was measured spectrophotometrically at 340 nm in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl, 5 mM-MgCl₂, 1.33 mM-ATP, 0.33 mM-phosphoenolpyruvate, 0.1 mM-fructose 1,6-bisphosphate, 0.16 mM-NADH and 25 μg of lactate dehydrogenase. The activity under these conditions was 40% of that described by Irving & Williams (1973) at pH 7.4 (L. G. Dann & H. G. Britton, unpublished work). Irving & Williams (1973) obtained a specific activity of 20 units/mg for their most active preparations. A value of 8 units/mg has therefore been assumed for the pure enzyme at pH 8.5.

Results

Exchange of phosphoenol[1-14C]pyruvate with pyruvate

Pyruvate kinase (0.37 unit) was incubated at 25°C with phosphoenol[1-14C]pyruvate (150 μM) and pyruvate (40 mm) in 2 ml of 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl, sufficient MgCl₂ to give 5 mM-MgCl₂ and tetraethylammonium chloride to give I 0.2. Samples (5 μl) were chromatographed on polyethyleneimine-cellulose and the phosphoenolpyruvate and pyruvate areas cut out and counted for radioactivity. After 14 or 15 min, 20 μl of an ADP solution was added to give a final concentration of 0.23 mM. No exchange of label (<1%) between phosphoenolpyruvate and pyruvate occurred, either in the presence of fructose 1,6-bisphosphate (150 μM) or in its absence, despite a rapid conversion of phosphoenolpyruvate into pyruvate after the addition of ADP (Figs. 1a and 1b). Experiments duplicating those illustrated gave similar results. A 1% conversion of phosphoenolpyruvate into pyruvate would correspond to a flux of phosphoenolpyruvate to pyruvate of 0.8 nmol/min per unit of enzyme. The rate of exchange is therefore less than 0.08% of the forward reaction measured under similar conditions. These experiments do not necessarily exclude a phosphoenzyme mechanism if the reaction was very nearly irreversible. However, in duplicate experiments similar to that illustrated in Fig. 1(a), ADP-Mg²⁺ and ATP-Mg²⁺ (final concn. 0.145 mM and 1.63 mM respectively) were added at 11 min to yield an equilibrium mixture of substrates and products. No exchange occurred before the addition of ADP-Mg²⁺ and ATP-Mg²⁺, but the rates of exchange after the addition were 133 and 109 nmol/min per unit. The exchange in the absence of ADP-Mg²⁺ and ATP-Mg²⁺ was thus less than 1% of the exchange in the presence of these substrates. For a Ping Pong mechanism, however, the exchange should have been more rapid in the absence of ADP-Mg²⁺ and ATP-Mg²⁺. Experiments were also carried out under conditions similar to those in Fig. 1(a) apart from the use of 50 mM-potassium phosphate buffer, pH 6.2. In duplicate experiments no exchange was observed, but the addition of ADP-Mg²⁺ and ATP-Mg²⁺ (final concn. 0.145 mM and 1.63 mM respectively) exchange occurred at 34 and 42 nmol/min per unit.

Attempts to prepare labelled phosphoenzyme

[³²P]Phosphoenolpyruvate (200 pmol) was incubated with 675 pmol of pyruvate kinase (2700 pmol of active centres, assuming four per molecule; Hubbard

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**Fig. 1. Exchange of phosphoenol[1-14C]pyruvate with pyruvate**

The experiments were conducted as described in the text, (a) in the presence of 150 μM-fructose 1,6-bisphosphate and (b) in the absence of fructose 1,6-bisphosphate. At the times denoted by the arrows, small volumes of ADP were added (final concn. 230 μM). Radioactivity detected as phosphoenol[1-14C]pyruvate (●) or as [1-14C]pyruvate (○) is expressed as c.p.m. per 5 μl sample. The recovery of [1-14C]pyruvate from the chromatogram was approx. 70% of the recovery of phosphoenol[1-14C]pyruvate.
Elution profile of a mixture of \(^{32}\text{P}\)phosphoenolpyruvate (0.2 nmol), fructose 1,6-bisphosphate (1.98 nmol) and pyruvate kinase (0.675 nmol) in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl and 5 mM-MgCl\(_2\), from a column of Sephadex G-25 as described in the text. Elution was monitored at 254 nm (---). Total \(^{32}\text{P}\) (○) and \(^{32}\text{P}\)Pi (◉) are given as c.p.m. per fraction (1 ml). The recovery of \(^{32}\text{P}\) from the column was >85%. Fractions 7 and 8 contained >90% of the enzyme activity applied to the column.

Discussion

The spectrophotometric data gave no evidence for the formation of a phosphoenzyme either at pH 8.5 or at pH 6.2. This result was confirmed by the gel-filtration experiments at pH 8.5. In these latter
experiments the amount of phosphoenzyme that might have been formed would depend on the equilibrium constant governing its formation, and a complete phosphorylation of the enzyme might not be expected. Nevertheless, the very high sensitivity inherent in the technique provides strong evidence excluding phosphoenzyme formation, and both of these techniques should have detected the formation of a phosphoenzyme even if it was unstable. However, the amount of enzyme present will have been overestimated if the value assumed for the specific activity of the pure enzyme should be low. This could explain the apparent lack of phosphoenzyme formation, but the error would have to be very large, especially since a large excess of enzyme was present in the gel-filtration experiments. Further, the lack of exchange of $^{14}$C between phosphoenolpyruvate and pyruvate at both pH 6.2 and 8.5 provides strong evidence to exclude phosphoenzyme formation, which does not depend on the amount of enzyme present. If is concluded therefore that it is unlikely that pyruvate kinase from rabbit liver has a phosphoenzyme mechanism.

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References


