Biochem. J. (1976) **153**, 127–134
Printed in Great Britain

Purification and Properties of Pyruvate Kinase from the Hepatopancreas of *Carcinus maenas*

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(*Received* 30 *June* 1975)

1. Pyruvate kinase from the hepatopancreas of the common shore crab, *Carcinus maenas*, was purified to a specific activity of 240 units/mg of protein in the assay conditions described. 2. In one method of purification the enzymic activity could be resolved into two fractions after chromatography on DEAE-cellulose. Fructose 1,6-diphosphate was able to effect the conversion of one form (peak 1) into the second (peak 2). 3. In the presence of a saturating concentration of fructose 1,6-diphosphate both forms of the enzyme were kinetically similar. 4. Polyacrylamide-gel electrophoresis of the enzyme 1 day after preparation showed a single protein band. On storage at least three protein bands became visible, all of which were associated with pyruvate kinase activity. 5. Chromatography of the enzyme on Sephadex G-200 indicated a mol.wt. of 247000, but in the presence of fructose 1,6-diphosphate the elution volume of the enzyme increased corresponding to a mol.wt. of 193000. 6. Dissociation of the enzyme in sodium dodecyl sulphate and 2-mercaptoethanol followed by polyacrylamide-gel electrophoresis produced one major protein band with a mol.wt. of 55000.

Studies on pyruvate kinase (EC 2.7.1.40) from a variety of sources have shown that there are basically two types of activity, one of which is stimulated by fructose 1,6-diphosphate, whereas the other is insensitive to this ligand. These have been designated type L and M respectively (Tanaka *et al.*, 1967). They can be distinguished on chromatographic, electrophoretic, immunological and kinetic properties. Normally, type L pyruvate kinase predominates in gluconeogenic tissues (Tanaka *et al.*, 1967).

The hepatopancreas of the common shore crab, *Carcinus maenas*, has been shown to be a gluconeogenic tissue (Toghrol, 1969) but few enzymes have been isolated from crustacean sources. Since the activity of the glycolytic enzyme, pyruvate kinase, is implicated in the regulation of these pathways it was decided to isolate this enzyme and compare its properties with those from the better documented systems.

The present paper reports the isolation of pyruvate kinase from the hepatopancreas of *C. maenas* and an investigation of some of its properties.

Experimental

Materials

Trizma base, fructose 1,6-diphosphate, EGTA,* NADH, sheep haemoglobin, bovine serum albumin (Cohn fraction V), horse heart cytochrome *c*, rabbit muscle pyruvate kinase and jack-bean urease (EC 3.5.1.5) were obtained from Sigma Chemical Co. Ltd. (London S.W.6, U.K.). ADP, phosphoenolpyruvate (tricyclohexylamine salt), pig heart lactate dehydrogenase (EC 1.1.1.27), rabbit muscle creatine kinase (EC 2.7.3.2) and bovine liver catalase (EC 1.11.1.6) were supplied by Boehringer Corp, Ltd. (London W.5, U.K.). Blue Dextran 2000 and Sephadex G-200 were purchased from Pharmacia, Uppsala, Sweden. Cellulose powder (CF11), DEAE-cellulose (DE32) and all other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

Animals

The common shore crab, *Carcinus maenas*, was collected locally from Southampton Water and kept in an aquarium at 10–12°C in circulating sea water collected from the same source.

Methods

Assay of pyruvate kinase activity. Pyruvate kinase was assayed by the method of Bücher & Pfleiderer (1955) except that the buffer used was 25mM-Tris/HCl, and 1mM-EGTA was added throughout. Reaction, which was linear with time, was initiated by the addition of pyruvate kinase, and was followed at 340nm and 25°C in a Pye–Unicam SP.1800 spectrophotometer coupled to a Pye–Unicam AR25 recorder. One unit of activity is defined as the amount of pyruvate kinase that produced 1 μmol of pyruvate/min at 25°C. Specific activity is defined as the number of units/mg of protein.

Preparation of acetone-dried powder. The hepatopancreases from 100–200 crabs were removed and placed in ice-cold 0.5M-NaCl. When sufficient material had been dissected the NaCl was decanted and the tissue homogenized in 10vol. of acetone, at −15°C, in a top-drive macerator for 3min. Acetone was removed by filtration on a Buchner funnel. This operation was repeated twice except that the homogenization was continued for 1min only. The powder was dried in a current of cold air and stored *in vacuo* over silica gel at −20°C until required.

Calcium phosphate gel. This was prepared by two separate methods. In enzyme preparation A (see the Results section) the method of Swingle & Tiselius (1951) was used. The gel was stored as a suspension in distilled water at a concentration of 50mg dry wt. of gel/ml. For use in column chromatography 1vol. of gel was mixed with 4vol. of a 10% (w/v) suspension of cellulose powder in distilled water. The calcium phosphate gel used in enzyme preparation B was prepared by the method of Mathews *et al.* (1964). Providing the gel was used within a few days of preparation it was not necessary to add cellulose powder to improve the flow rate.

Chromatography on Sephadex G-200. Molecular weights were determined as described by Andrews (1964) except that the gel was equilibrated with 50mM-Tris/HCl buffer, pH7.4. The calibration curve was constructed with cytochrome *c*, pyruvate kinase, lactate dehydrogenase, creatine kinase, urease and Blue Dextran.

Chromatography on DEAE-cellulose. Before use the DEAE-cellulose was pre-cycled as recommended by the manufacturers. All chromatography, except the gel filtration, was performed with a solvent pressure generated by a Medcalf Hyflow pump (model C). In this way flow rates of 30–50ml/h were obtained in spite of the presence of 33% (v/v) glycerol.

Data analysis. The results of the kinetic experiments were fitted to the Hill equation by a least-squares hyperbolic regression analysis based on the method of Atkins (1973) using a Hewlett–Packard 9810 desk-top computer.

Polyacrylamide-gel electrophoresis. A Shandon analytical polyacrylamide-gel electrophoresis apparatus was used throughout. The gels contained 10% (w/v) acrylamide with the ratio of cross-linker/monomer constant at 3:111. A continuous buffer system was used in which both the electrode compartments and the gels contained Tris/glycine buffer (2.4g of Tris and 11.52g of glycine/l). The gels were pre-cooled to 4°C before use and maintained at this temperature during electrophoresis. Protein was put on to the gels in aq. 33% glycerol and run at a constant current of 2mA/tube for twice the elution time of Bromophenol Blue; no protein was eluted in this time. The gels were stained in 0.3% (w/v) Coomassie Blue for at least 2h (Weber & Osborn, 1969). Excess of stain was removed in a Shandon transverse gel de-stainer after which the gels were scanned in a Joyce–Loebl u.v. gel scanner at 265nm. Pyruvate kinase activity was measured in a duplicate gel sliced at 2mm intervals. Each slice was extracted into 0.1ml of 30mM-phosphate buffer/aq. 33% (v/v) glycerol, pH7.4, for 1h at 4°C. Enzyme activity in the extracts was determined as described above, except that the assay included 0.5mM-fructose 1,6-diphosphate.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Both continuous (Weber & Osborn, 1969) and discontinuous (Neville, 1971) buffer systems were used, together with 10% (w/v) gels. The running pH of the Neville (1971) system used was 9.5. *C. maenas* pyruvate kinase was dissociated in 10mM-phosphate buffer, pH7.0, in the presence of sodium dodecyl sulphate and 2-mercaptoethanol at 37°C as indicated in the text. Molecular weights were estimated by comparing the mobility of the fragments with that of the subunits of standard proteins (bovine serum albumin, pyruvate kinase, lactate dehydrogenase, haemoglobin and cytochrome *c*).

Dialysis. Before use dialysis tubing was boiled for several hours in 1mM-EDTA, pH7.4. The latter was changed several times during the process, after which the tubing was stored in 1mM-EDTA and washed with distilled water before use.

Preparation of buffers. All buffers used in the preparation of the enzyme, including those used for column chromatography, contained 33% (v/v) glycerol in aqueous solution.

Protein determination. In the early stages of protein purification the biuret method (Gornall *et al.*, 1949) was used. In the later stages when the protein concentration was low either the ninhydrin procedure of Hirs (1967) or the method of Lowry *et al.* (1951) was used. Previously dried bovine serum albumin (Cohn fraction V) was used as the primary standard. Interference by glycerol in the Lowry *et al.* (1951) procedure was allowed for by dissolving the standard protein in aq. 33% (v/v) glycerol.

Results

Stability of pyruvate kinase

Extraction of the acetone-dried powder in an aqueous buffer medium resulted in less than 10% of the original activity remaining after 18h storage at 0°C. The addition of substrates and/or products, with or without thiol reagents, or the addition of the protease inhibitor phenylmethanesulphonyl fluoride, did not improve the stability of the crude extract. By contrast the use of aqueous glycerol buffers gave a recovery of 90% when the extract was stored under the same conditions. Again, the addition of substrates, products or protease inhibitor did not increase the stability. Storing the purified enzyme (preparation A

or B) in 33% (v/v) glycerol buffer at −20°C resulted in retention of activity. Indeed enzyme purified by preparation B has been stored for over 18 months with no loss of activity.

Effect of buffers

In common with pyruvate kinase from other sources ionic strength of the assay medium had a pronounced effect on the activity of the enzyme (Melchoir, 1965; Llorente *et al.*, 1970). Increasing the concentration of Tris/HCl buffer in the assay from 12.5 to 50mM decreased activity by 40%. Phosphate buffer was more inhibitory than a similar concentration of Tris/HCl buffer, and Tris/maleate buffer more so than phosphate buffer.

Since calcium phosphate gel was used in the preparation of the enzyme, and Ca^{2+} inhibits the enzyme (in our hands, 8.3μM-Ca^{2+} gave a 20% inhibition), the effect of EGTA was investigated. In the absence of Ca^{2+} and the presence of 80μM-phosphoenolpyruvate, increasing concentrations of EGTA increased the activity of pyruvate kinase. This effect was noticeable above 1mM. At 100mM-EGTA no further increase in activity was observed. However, concomitant with this change the enzyme was no longer stimulated by the addition of 10μM-fructose 1,6-diphosphate. As a consequence, the assay of pyruvate kinase described in the Experimental section was used as a routine. The presence of 1mM-EGTA was sufficient to produce linear reaction rates after calcium phosphate treatment of the enzyme, but it did not decrease the stimulation of pyruvate kinase activity caused by the addition of fructose 1,6-diphosphate. EDTA had a similar effect but high concentrations could not be used since it removed Mg^{2+} which is essential for enzyme activity.

Purification of pyruvate kinase (preparation A)

All operations were carried out at 0–4°C unless otherwise stated. Acetone-dried powder (70g) was stirred for 30min in 800ml of 67mM-Tris/HCl buffer containing 0.67mM-EDTA and 1mM-2-mercaptoethanol. The extract was centrifuged for 30min at 15000*g* (MSE High-Speed 18 centrifuge, 6×250ml head, 12000rev./min) and the supernatant was adjusted to pH5.0 with 1M-acetic acid with continuous stirring. The precipitate was stirred for a further 15min then removed by centrifugation at 15000*g* for 15min and the supernatant cooled to −5°C. Ethanol, at −20°C, was added slowly to a final concentration of 35% (v/v). The suspension was stirred for 15min then centrifuged at 15000*g* for 15min and the precipitate discarded. Calcium phosphate gel suspension was added to the supernatant until 5–10% of the activity had been removed. The gel was removed by centrifugation at 10000*g* for 5min. Addition of gel suspension to the supernatant was continued until only 10–15% of the activity remained in solution. The gel was collected by centrifugation and washed with 3×20ml portions of 20mM-potassium phosphate buffer, pH7.4 (no glycerol). Enzyme activity was eluted from the gel by resuspending in 500mM-potassium phosphate buffer, pH7.4 (no glycerol). This was repeated until little activity remained on the gel. The total volume was usually between 70–90ml. Glycerol was added to the extract to a final concentration of 33% (v/v). De-Acidite FF (OH^-) and Zeo-Karb 225 (H^+) ion-exchange resins (1:1, by wt.) were added to the solution until the pH dropped to 6.0; this decreased the phosphate concentration to approximately 10mM. After filtration through gauze to remove the resin, the protein solution was put on a calcium phosphate/cellulose column (2.5cm×21cm) equilibrated with 10mM-potassium phosphate buffer, pH6.0. Two void volumes of 50mM-potassium phosphate, pH7.4, were passed through the column, after which the activity was eluted with 150mM-potassium phosphate, pH7.4, and the eluate collected in 3ml fractions. The most active of these were pooled and deionized with resin (as described above) and put on a DEAE-cellulose column (2.5cm×8cm)

Table 1. *Summary of the purification of pyruvate kinase from hepatopancreas (preparation A)*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Initial extract	750	10800	3200	0.29
pH5.0	750	6500	3200	0.49
35% ethanol	1065	5300	3000	0.56
$Ca_3(PO_4)_2$ (batchwise)	95	1280	2020	1.6
$Ca_3(PO_4)_2$ column	66	350	733	21.0
DEAE-cellulose column				
Peak 1	4*	1.01†	131	129.0
Peak 2	4*	4.90†	208	42.5

* These values were obtained after concentration in a pressure cell.
† Protein determined by the Lowry *et al.* (1951) method.

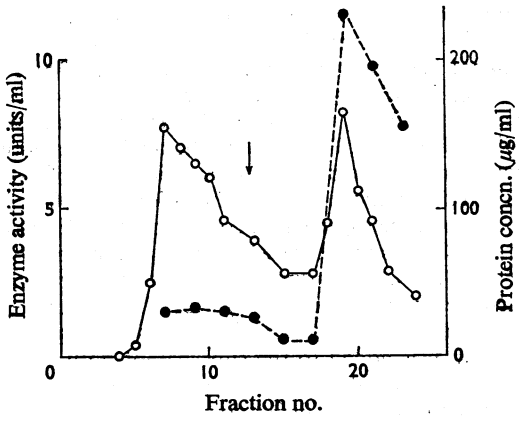

Fig. 1. *Elution profile from DEAE-cellulose during purification of C. maenas pyruvate kinase by method A*

The complete chromatographic conditions are given in the text. Fraction 1 was the first collected after the ionic strength of the elution buffer was increased by the addition of KCl to 100mM. At the arrow further KCl was added to a total concentration of 150mM. The fraction size was 3.6ml and protein (●) was measured by the Lowry *et al.* (1951) method. ○, Enzyme activity.

equilibrated with 10mM-phosphate buffer, pH6.0. After washing with 2 void volumes of 30mM-phosphate buffer, pH7.4, containing 50mM-KCl to remove unwanted protein, the concentration of KCl was increased to 100mM. This resulted in the appearance of enzyme activity in the eluate (peak 1). Finally, when the concentration of KCl in the buffer was increased to 150mM more activity appeared in the eluate (peak 2) (see Fig. 1). A summary of the purification procedure is shown in Table 1.

The elution profile of pyruvate kinase activity from DEAE-cellulose (Fig. 1) clearly shows the presence of two peaks of activity. Even after prolonged washing with the first elution buffer complete separation of the peaks could not be achieved. Determination of the stimulation in activity of the enzyme in peaks 1 and 2 caused by 0.5mM-fructose 1,6-diphosphate showed that peak 1 enzyme was increased up to eightfold whereas that in peak 2 was less than 1.2-fold, when the phosphoenolpyruvate concentration was 80μM.

To see if there was a dynamic relationship between the enzyme in peaks 1 and 2, the two most active fractions from peak 1 were pooled, deionized with resin, and divided into two equal parts. To one was added 15μM-fructose 1,6-diphosphate. The fractions were rechromatographed separately on two identical columns (1.0cm×3.8cm) of DEAE-cellulose equilibrated and eluted as described in preparation A except that one column always had 15μM-fructose

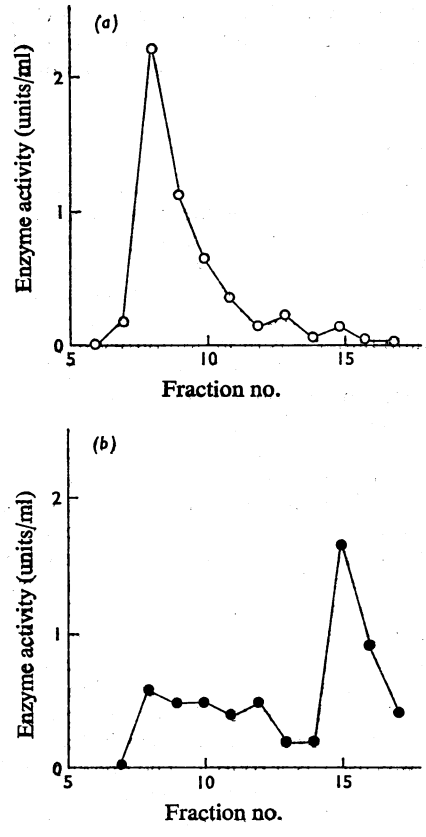

Fig. 2. *DEAE-cellulose elution profile showing conversion of peak 1 into peak 2 on addition of fructose 1,6-diphosphate*

Two identical DEAE-cellulose columns were run simultaneously after deionized enzyme, previously eluted as peak 1, had been applied. (*a*) Control experiment: a volume of water equal to the volume of fructose 1,6-diphosphate added in (*b*) was added to the enzyme. No fructose 1,6-diphosphate was present during the elution. (*b*) Fructose 1,6-diphosphate (15μM) was added to the enzyme immediately before chromatography on a column pre-equilibrated with buffer containing 15μM-fructose 1,6-diphosphate. This concentration of fructose 1,6-diphosphate was maintained throughout the elution. Fraction 3 was the first collected after increasing the KCl content of the buffer to 50mM. From fraction 7 the KCl concentration was 100mM and from fraction 13 the buffer was 150mM in respect to KCl, 2ml fractions were collected.

1,6-diphosphate present in the buffers (Fig. 2). Under these conditions, enzyme that had chromatographed in peak 1, contained a substantial proportion of enzyme that now chromatographed in the position of peak 2 when fructose 1,6-diphosphate was present. In the absence of fructose 1,6-diphosphate there was

Table 2. *Summary of the purification of pyruvate kinase from hepatopancreas (preparation B)*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Initial extract	920	8750	4000	0.46
pH5.0	950	5900	3750	0.64
33–46% ethanol fractionation	61	715	2030	2.9
$Ca_3(PO_4)_2$ column	108	26	1070	41.0
Post dialysis	110	26	505	19.4
DEAE-cellulose column	3*	1.13†	450	398.0

* These values were obtained after concentration in a pressure cell.
† Protein was determined by the Lowry *et al.* (1951) method. The ninhydrin protein determination gave a total protein content of 1.66mg and thus a specific activity of 240.

no such change in chromatographic properties. The total recovery of enzyme was 70% in the presence of fructose 1,6-diphosphate and 67% in its absence.

Attempts to identify a specific ligand that would convert peak 2 enzyme into that of peak 1 were unsuccessful. However, re-chromatography of peak 2 enzyme alone on DEAE-cellulose caused a significant appearance of enzyme that was eluted in the position of peak 1. An experiment conducted in the presence of the allosteric inhibitor L-alanine (Giles *et al.*, 1975) instead of the activator fructose 1,6-diphosphate in the manner described in the paragraph above, but using enzyme from peak 2, showed that the presence of L-alanine had no significant effect on the formation of peak 1 enzyme.

From these results it is possible that the two enzyme peaks are conformers of the same protein. In an effort to substantiate this suggestion initial-rate studies in the presence and the absence of 0.5mM-fructose 1,6-diphosphate on the enzyme in peaks 1 and 2 were undertaken.

In the absence of fructose 1,6-diphosphate enzyme from both peaks had Hill coefficients greater than 1 when phosphoenolpyruvate was the variable substrate, but of 1 when ADP was varied. The $s_{0.5}$ (phosphoenolpyruvate), and its associated Hill coefficient, decreased as the concentration of ADP was increased. In the presence of 0.5mM-fructose 1,6-diphosphate increasing the ADP concentration from 71 to 225μM gave $s_{0.5}$ (phosphoenolpyruvate) values of 35 and 32μM respectively for the enzyme from peak 1. The corresponding values for peak 2 enzyme were 37 and 31μM when the concentration of ADP increased from 79 to 230μM respectively. In the presence of fructose 1,6-diphosphate the $s_{0.5}$ (ADP) values were 86 and 95μM for peak 1 and peak 2 enzymes respectively. The similarity of these kinetic constants further supports the idea that the two peaks of pyruvate kinase activity obtained after DEAE-cellulose chromatography are different conformers of the same protein. It was observed that peak 2 enzyme had a high E_{260}/E_{280} ratio, indicating that some non-protein material was present. One possibility was that this was nucleic acid that had not been removed. An ethanol-precipitation stage was introduced, decreasing the ratio to 0.71, but altering the ion-exchange properties of the enzyme (preparation B).

Purification of pyruvate kinase (preparation B; Table 2)

The initial steps, up to and including adjustment to pH5.0, were identical with those for preparation A except that the acetone-dried powder was extracted into 1 litre of buffer. Ethanol, at −20°C, was added to the supernatant from the pH5-adjustment step and the protein precipitated between 33 and 46% (v/v) ethanol was collected by centrifugation after the solution had been stirred for at least 20min at −10°C after each addition of ethanol. The pellet was resuspended in 10mM-potassium phosphate buffer, pH6.0, and clarified by centrifugation at 34000*g* for 15min (8×50ml head, 18000rev./min). The clear yellow–green solution was placed on a column (2.5cm×21cm) of calcium phosphate gel equilibrated with the buffer in which the protein was dissolved.

Elution of unwanted protein with 50mM-potassium phosphate buffer, pH7.4, was continued until the eluate was protein-free. This was followed by 1 void volume of 90mM-phosphate buffer, which resulted in the elution of less than 10% of the total activity. The bulk of the enzyme activity was eluted in 140mM-phosphate buffer, pH7.4, and collected in 3ml fractions. Those with an activity greater than 5 units/ml were pooled and the enzyme (80–100ml) was dialysed against 3 litres of 2mM-potassium phosphate buffer, pH6.0, containing 33% (v/v) glycerol. After dialysis the phosphate content of the protein solution was 10–15mM. The dialysed solution was put on a column (2.5cm×8.0cm) of DEAE-cellulose equilibrated with a similar buffer to that used for dialysis. After washing with 30mM-phosphate

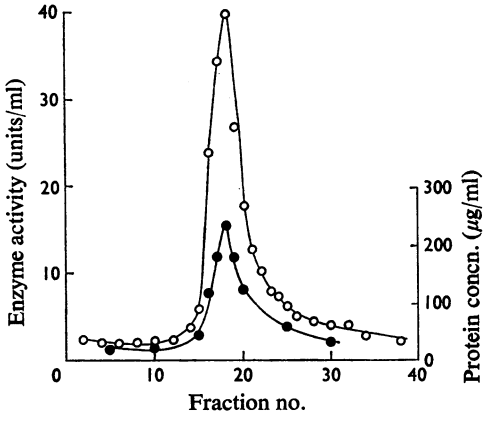

Fig. 3. *Elution profile from DEAE-cellulose during the purification of C. maenas pyruvate kinase by method B*

The complete chromatographic conditions are given in the text. Fraction 4 was the first collected after increasing the KCl content of the elution buffer from 100 to 150mM. The fraction size was 3ml and protein (●) was measured by the Lowry *et al.* (1951) method. ○, Enzyme activity.

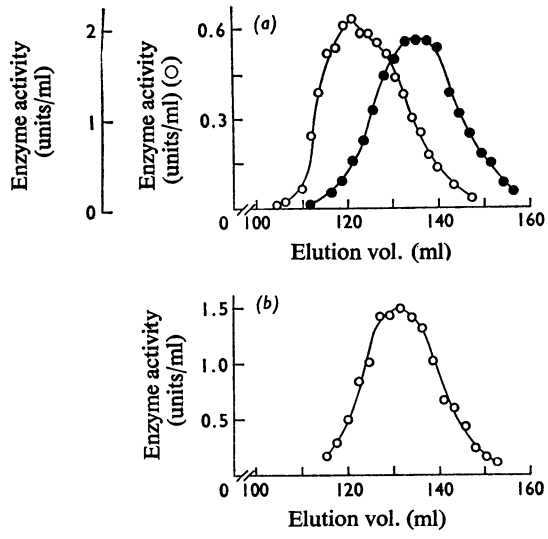

Fig. 4. *Molecular-weight determination of C. maenas pyruvate kinase by gel filtration*

The elution profiles from Sephadex G-200 for enzyme purified by method B are shown. Native enzyme alone (*a*, ●), in the presence of 20mM-EGTA (*a*, ○), or 25μM-fructose 1,6-diphosphate (*b*).

buffer, pH7.4, and a similar buffer containing 50mM-KCl until the eluate was protein-free, the concentration of KCl in the buffer was increased to 100mM and 2 void volumes of buffer were passed through the column. Finally, the enzyme was eluted as a single peak when the total concentration of KCl in the buffer was 150mM (Fig. 3). Active fractions were pooled and concentrated in a pressure cell fitted with an Amicon PM 10 membrane. The concentrated enzyme was stored at −20°C until required.

Estimation of the specific activity of the enzyme depended on the protein assay used. Protein concentration was higher in the ninhydrin assay than in the Lowry *et al.* (1951) method. Since the latter assay depends on the presence of aromatic amino acids in the protein this result presumably reflects a lower-than-average content of these amino acids in the enzyme. Such a situation is known to occur in pyruvate kinase prepared from other sources (Cottam *et al.*, 1969; Kuczenski & Suelter, 1970; Bischofberger *et al.*, 1970; Kutzbach *et al.*, 1973). The higher specific activity of enzyme from preparation B compared with either peak 1 or peak 2 from preparation A is not unreasonable as a further purification step has been included..

At low concentrations of phosphoenolpyruvate the activity of the enzyme was stimulated 2.5–3.5 times by the addition of 500μM-fructose 1,6-diphosphate. Initial-rate studies showed that in the presence of this concentration of fructose 1,6-diphosphate the apparent K_m for phosphoenolpyruvate was 49 and 36μM when the ADP concentration was 79 and 226μM respectively. The apparent K_m for ADP was 119μM at a phosphoenolpyruvate concentration of 196μM. These values are similar to those obtained for pyruvate kinase purified by preparation A. Thus the same protein is produced by both methods of preparation.

Electrophoresis of the enzyme on 10% polyacrylamide gels 1 day after preparation showed one major band, with a faintly staining band just penetrating the gel also present. After 4 weeks' storage at least three faster-moving protein bands appeared, all of which were associated with enzyme activity.

Sephadex G-200 gel chromatography

After gel filtration the enzyme activity assays were non-linear with time under the conditions described in the Experimental section. However, if the reaction was initiated with phosphoenolpyruvate after the enzyme had been pre-incubated for 5min with the ADP and 0.5mM-fructose 1,6-diphosphate the time-course of the reaction was linear. The qualitative elution profile was independent of the assay procedure used, though the apparent recovery of enzyme increased when the assay involving preincubation was used. The results of chromatographing the enzyme alone, with 25μM-fructose 1,6-diphosphate and with 20mM-EGTA are shown in Fig. 4. This shows clearly that the elution volume is increased when fructose 1,6-diphosphate or EGTA is present, indicating a

decrease in molecular weight. The elution volumes obtained correspond to 247000, 193000 and 170000–190000 mol.wt. for enzyme chromatographed alone, with fructose 1,6-diphosphate and with EGTA respectively.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

Initial attempts to determine the molecular weight of the enzyme subunit after incubation in 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol for up to 20h followed by electrophoresis in a continuous buffer system produced gels with up to 12 bands, the most prominent of which had a mol.wt. of 85000. Increasing the time of incubation with sodium dodecyl sulphate and 2-mercaptoethanol increased the lower-molecular-weight bands at the expense of the largest. The bands obtained were rather diffuse, a characteristic of continuous buffer systems (Neville, 1971), which leads to some uncertainty in the mobility of the protein. The rapid dissociation to the 85000-mol.wt. fragment, followed by a slower breakdown could be caused by a fast initial dissociation followed by a slower second phase yielding the true subunit. Previous workers have experienced similar difficulties in analysing the subunit composition of pyruvate kinase from yeast and pig liver (Bischofberger *et al.*, 1971), where the subunits are formed only in strongly dissociating conditions. Another possibility is the presence of trace amounts of a protease that acts on the subunit over the 20h incubation period to give smaller fragments (Pringle, 1970).

More rigorous dissociation of the *C. maenas* enzyme in 1% (w/v) sodium dodecyl sulphate and 10% (v/v) 2-mercaptoethanol over a short time-course (3.5h), followed by electrophoresis in the high-resolution discontinuous buffer system of Neville (1971), produced gels showing only one major band, with a mol.wt. of 55000, and one minor band, with a mol.wt. of 70000. From densitometer traces it was estimated that the minor band contained 10% of the total protein stain present. Since exhaustive tests on the purity of the enzyme preparation have not been completed the possibility that the minor component could be derived from contaminating proteins cannot be ruled out.

Discussion

Pyruvate kinase prepared from frog heart exists in two interconvertible forms which differ in their sensitivity to fructose 1,6-diphosphate (Flanderers *et al.*, 1971). This situation is analogous to that with pyruvate kinase purified from the hepatopancreas of *C. maenas* in preparation A. The enzyme eluted from DEAE-cellulose by 100mM-KCl (peak 1) was markedly stimulated by fructose 1,6-diphosphate whereas the enzyme eluted by 150mM-KCl (peak 2) was relatively insensitive to the presence of this ligand. Conversion of peak 1 enzyme into peak 2 was readily effected by the addition of fructose 1,6-diphosphate whereas a specific ligand causing the reverse transformation could not be identified because re-chromatography of peak 2 enzyme alone on DEAE-cellulose resulted in the appearance of a significant proportion of peak 1 enzyme which masked any possible effect caused by the ligand. Activation by fructose 1,6-diphosphate is a property that this *C. maenas* pyruvate kinase has in common with the mammalian type L enzyme present in gluconeogenic tissues.

Initial-rate measurements in the presence of fructose 1,6-diphosphate showed that, within experimental error, the $s_{0.5}$ values for the substrates for each form of pyruvate kinase were similar. This provides strong evidence that peaks 1 and 2 are different conformers of the same protein species. The fact that the alternative method of purification (preparation B) resulted in only one form of the enzyme, which eluted from DEAE-cellulose under conditions similar to those for peak 2 (preparation A), might be explained by the precipitation of the enzyme with ethanol, a step that was absent in the latter preparation.

Isoelectric focusing of the pig liver type L pyruvate kinase during purification (Hess & Kutzbach, 1971) showed heterogeneity of the enzyme. The ratios between the various forms altered with the stage of purification, as did the number of forms present. It is possible, therefore, that in the purification of the *C. maenas* enzyme inclusion of the ethanol-precipitation step has a similar effect that is reflected in the ion-exchange chromatography. Different, but interconvertible forms could also explain the observation that enzyme prepared by method B was eluted from DEAE-cellulose under similar conditions to those of peak 2 (preparation A), yet it is stimulated by fructose 1,6-diphosphate whereas peak 2 is barely affected.

In our hands EGTA mimicked the effect of fructose 1,6-diphosphate at a low concentration of phosphoenolpyruvate (80μM). Increasing the concentration of EGTA up to 1mM had little effect on the fructose 1,6-diphosphate activation of the enzyme. At higher concentrations enzyme activity increased, but with a concomitant decrease in fructose 1,6-diphosphate stimulation. In 100mM-EGTA there was a complete loss of fructose 1,6-diphosphate stimulation, though the maximum activities found with either EGTA or fructose 1,6-diphosphate were similar. Qualitatively, EDTA had a similar effect, but high concentrations could not be used since this ligand chelates Mg^{2+} which is essential for activity. These results are the reverse of those obtained by Bailey *et al.* (1968) who found that preincubation of rat liver pyruvate kinase in the presence of EDTA caused an increase in

co-operativity with respect to phosphoenolpyruvate and fructose 1,6-diphosphate. Pogson (1968) using a preparation of pyruvate kinase from rat adipose tissue observed a similar increase in co-operativity. However, both authors were using a relatively crude preparation of the enzyme.

Filtration of pyruvate kinase on Sephadex G-200 gave an elution volume that corresponded to a mol.wt. of 247000, but in the presence of fructose 1,6-diphosphate a value of 193000 was obtained. With EGTA present in the elution buffer the peak of the enzyme elution profile was less well-defined but corresponded to a mol.wt. of 170000–190000. Since polyacrylamide-gel electrophoresis of pyruvate kinase after dissociation in sodium dodecyl sulphate and 2-mercaptoethanol, gave a possible subunit mol.wt. of 55000 the decrease in molecular weight of the native protein approximates to the loss of one subunit. More data is required to elucidate the polymeric structure of the protein since the above results suggest that *C. maenas* pyruvate kinase contains either four or five subunits per molecule but only three or four in the presence of fructose 1,6-diphosphate.

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