A Kinetic Study of Pig Liver Pyruvate Kinase Activated by Fructose Diphosphate

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The paper reports a study of the reaction between phosphoenolpyruvate, ADP and Mg^{2+} catalysed by pig liver pyruvate kinase when activated by fructose diphosphate and K^+ . The experimental results are consistent with two non-sequential mechanisms in which the substrates and products of the reaction are phosphoenolpyruvate, ADP, Mg^{2+} , pyruvate and MgATP. Pyruvate release occurs before ADP binding. Two Mg^{2+} ions are involved, though the two Mg^{2+} -binding sites cannot be occupied simultaneously. An isomerized enzyme complex forms before release of MgATP. Values were determined for the Michaelis constants of the reaction. Apparent MgATP inhibition constants are also given.

The glycolytic enzyme pyruvate kinase (EC 2.7.1.40) exists in at least two forms in liver. Thus Tanaka *et al.* (1967) have isolated M and L forms from rat liver. The L-type enzyme shows a sigmoidal relationship of initial velocity to phosphoenolpyruvate concentration unless activated by fructose diphosphate, when the relationship takes a non-inflected form and the affinity for phosphoenolpyruvate is enhanced. Balinsky *et al.* (1972) have also isolated two types of pyruvate kinase from normal human liver and from human hepatoma tissue. They showed that the L-type enzyme predominates in normal tissue and that its kinetic mechanism is ordered, with phosphoenolpyruvate binding before ADP. This mechanism is similar to that suggested by Macfarlane & Ainsworth (1972) for the yeast enzyme, which also requires activation by fructose diphosphate, but contrasts with the mechanism of the rabbit muscle enzyme, which is equilibrium random-order in type (Mildvan & Cohn, 1966; Ainsworth & Macfarlane, 1973). Two pyruvate kinases have been isolated from pig liver by Hess & Kutzbach (1971), who examined their properties by isoelectric focusing. It appears that pig liver contains an L-type enzyme which can exist in free and fructose diphosphate-bound forms. Hess & Kutzbach (1971) also showed that the bound L type is the major constituent of pyruvate kinase in crude liver extracts and that the free L type increases in proportion as purification proceeds.

This paper reports a study of L-type pig liver pyruvate kinase, purified 1000-fold. To facilitate comparison with previous studies of the rabbit muscle and baker's yeast enzymes (Ainsworth & Macfarlane, 1973; Macfarlane & Ainsworth, 1972), experiments were conducted with the enzyme in solutions kept at 25°C and pH 6.2 and containing

saturation concentrations of K^+ and fructose diphosphate throughout.

Experimental

Materials

ATP, ADP, NADH (all as sodium salts), phosphoenolpyruvate (cyclohexylammonium salt) and rabbit muscle lactate dehydrogenase (15371 ELAC) were products of Boehringer Corp. (London) Ltd., London W.5, U.K. Tetrapropylammonium hydroxide was supplied by Eastman Kodak Co., Rochester, N.Y., U.S.A. Sephadex G-25, G-200 and DEAE-Sephadex were obtained from Pharmacia, Uppsala, Sweden; hydroxyapatite was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All other reagents were either AnalaR or reagent grade as supplied by British Drug Houses Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Preparation of L-type pyruvate kinase from pig liver

Batches (200g) of pig liver were homogenized with 400ml of 10mM-phosphate (KH_2PO_4) buffer, pH 7.1, containing 150mM-KCl, 5mM- $MgSO_4$ and 1mM-EDTA in a Waring Blendor at 3600 rev./min for 1 min. The homogenization and all subsequent steps were conducted at 4°C. The homogenate was centrifuged for 30 min at 8000g in a Sorvall refrigerated centrifuge (GSA rotor) and the supernatant filtered through glass wool to remove fatty material. Then 144g of $(NH_4)_2SO_4$ was added to each litre of supernatant (25% saturation) with rapid stirring and the pH adjusted to 7.1 with 2M-KOH. The mixture was left for 30 min and then

centrifuged at 8000g for 30min. A further 123 g of $(\text{NH}_4)_2\text{SO}_4$ was added to each litre of the resulting supernatant (45% saturation); after adjustment to pH7.1, the mixture was left for 12h before centrifugation at 8000g for 30min. The precipitate was suspended in a minimum volume of 10mM-phosphate buffer, pH7.1, containing either 1mM-dithiothreitol (preparation 1) or 10mM-mercaptoethanol (preparation 2). It was then dialysed against this buffer solution for 12h. The enzyme was further purified by acetone precipitation between 25 and 45% (v/v) and successive gradient chromatography on DEAE-Sephadex and hydroxyapatite as described by Hess & Kutzbach (1971).

The kinetic study used two preparations of pyruvate kinase, preparations 1 and 2 with specific activity of 70 and 140 units/mg respectively at 25°C. Protein concentrations were determined from absorbance measurements at 280nm by using the value of $E_{1\text{cm}}^{0.1\%}$ given below. The preparations were stored as suspensions in 10mM-phosphate buffer, pH7.1, which was 45%-satd. with $(\text{NH}_4)_2\text{SO}_4$ and which contained either 1.0mM-dithiothreitol (preparation 1) or 10mM-mercaptoethanol (preparation 2).

Both preparations were examined by polyacrylamide-gel electrophoresis as described by Hunsley & Suelter (1969a). In both, the protein migrated as three bands with all the enzyme activity associated with the major staining band. No change in the position or number of the bands was observed when enzyme was used that had been incubated with 0.2mM-fructose diphosphate at 4°C for 24h before electrophoresis, although half the activity of the enzyme was lost during the incubation.

An attempt was made to purify the enzyme further by chromatography on Sephadex G-200. No significant increase in specific activity resulted, but half the activity of the enzyme was lost.

A small sample of pyruvate kinase (preparation 2) was dialysed against 1mM-Tris-HCl buffer, pH7.5. It was made up to 1.5ml with the same buffer and centrifuged to remove a small precipitate. The supernatant (1.23ml) had an E_{280} of 0.727. After drying at 110°C and cooling over P_2O_5 , the residue weighed 1.110mg. The same volume of buffer solution, when dried as above, gave a residue of 0.275mg. From these data an $E_{1\text{cm}}^{0.1\%}$ value of 1.07 was calculated, and used subsequently to determine pyruvate kinase concentrations.

Preparation of substrates, products and enzymes for use

Na^+ ions were removed from substrates and products and NH_4^+ ions from lactate dehydrogenase as described by Ainsworth & Macfarlane (1973). NH_4^+ ions were not removed from the samples of pyruvate kinase because so little was available: however, the maximum concentration of NH_4^+ ions

introduced into the assay along with the enzyme was only 3mM. At this concentration, and in comparison with 100mM-KCl also present, the effects of NH_4^+ ions may be neglected (Hunsley & Suelter, 1969b). Pyruvate kinase was therefore prepared for use by dissolving a small portion of the $(\text{NH}_4)_2\text{SO}_4$ suspension in 0.1M-Tris-HCl buffer, pH7.0, at 4°C to give an enzyme concentration of 0.1mg/ml. Preliminary experiments showed that the enzyme was stable for at least 2h under these conditions. Initial velocities were shown to be proportional to enzyme concentration.

The purity and concentrations of substrates were determined as described by Ainsworth & Macfarlane (1973).

Enzyme assays

Reaction mixtures contained, in a total volume of 1ml, tetrapropylammonium cacodylate buffer (0.1M in cacodylate), pH6.2, KCl (100 μmol), NADH (0.15 or 0.015 μmol) and fructose diphosphate (0.2 μmol) with substrates and sometimes a single product at the concentrations indicated in the Figures. Initial-velocity and ATP-product-inhibition studies were performed by using lactate dehydrogenase as a coupling enzyme (Bücher & Pfeleiderer, 1955). It was shown that the coupling enzyme was not rate-limiting by performing sample assays, covering the range of reactant concentrations used, in which the coupling enzyme was at one-half and twice the concentration stated in the Figures; no difference in the recorded initial velocity was observed.

After addition of the necessary components, the reaction mixtures were incubated for 5min at 25°C before addition of NADH and the coupling enzyme. The reactions were initiated by adding 10 μl or less of pyruvate kinase and the initial velocities estimated from the linear slopes of progress curves recorded by a Gilford 240 recording spectrophotometer at 340nm and with full scale set at either 1.0 or 0.1 absorbance unit.

Determination of substrate concentrations

The substrates of pyruvate kinase, phosphoenolpyruvate, ADP and Mg^{2+} , interact in substrate-level equilibria. It is therefore necessary to determine the concentrations of the different possible substrates by using the known equilibrium constants for their interconversions. This has been done, at given free Mg^{2+} concentrations, by the method described by Macfarlane & Ainsworth (1972) assuming that the dissociation constants do not change with ionic strength over the range of our study (Macfarlane *et al.*, 1974). As a result, we have evaluated *A* and *B* as the concentrations of phosphoenolpyruvate and

ADP, respectively, that are not complexed by Mg^{2+} (though K^+ may be bound), α and β as the corresponding Mg^{2+} -complexed forms and A_t and B_t as their total concentration. C is also defined as the given free Mg^{2+} concentration, and C_t as the total Mg^{2+} .

Computer analysis of data

Analysis of data was done with the computer programs of Cleland (1963b) after a graphical check had been made that the double-reciprocal plots were linear. Initial-rate data for uninhibited reactions at fixed free Mg^{2+} concentrations were analysed by the NONSEQUEN program, a copy of which was kindly made available to us by Dr. E. Heyde (School of General Studies, Australian National University, Canberra, Australia). The secondary slopes and intercepts thus obtained, together with their standard errors, were fitted as a linear function of the reciprocal free Mg^{2+} concentration by a weighted linear regression (Wilkinson, 1961; Ainsworth & Macfarlane, 1973). The data for inhibited reactions were analysed by the NONCOMP program. The data of Fig. 7 were analysed by the SEQUEN program.

Results

Effect of fructose diphosphate on the initial rate

Fig. 1 shows the dependence of the initial velocity on the concentration of fructose diphosphate. It is evident that the response reaches a plateau at 0.2mm-fructose diphosphate; this concentration of fructose diphosphate was therefore present in all the assay mixtures used in this study. It was shown that this concentration of fructose diphosphate was also saturating at the lowest concentrations of K^+ and Mg^{2+} ions, ADP and phosphoenolpyruvate used, by performing assays in which the concentration of fructose diphosphate was 0.1, 0.2 and 0.4mm. The same result was obtained with all other reactant concentrations that were sampled within the range of this study. It was also shown, in a separate experiment, that 2 μ M-fructose diphosphate would effect an identical enhancement in rate (after correction for loss of specific activity) if it was incubated with the enzyme for 24h before the initial rate was determined: under these conditions the further addition of 0.2mm-fructose diphosphate immediately before reaction commenced had no effect on the rate.

Identification of the substrates of pyruvate kinase

Macfarlane & Ainsworth (1972) proposed a test to identify the substrates of a kinase which requires the dependence of the reciprocal initial velocity on

Fig. 1. Effect of fructose diphosphate on the initial rate of the forward reaction catalysed by pig liver pyruvate kinase

The reaction mixture contained tetrapropylammonium cacodylate buffer, pH 6.2 (100 μ mol), KCl (100 μ mol), $MgCl_2$ (24 μ mol), ADP (5 μ mol), phosphoenolpyruvate (2 μ mol), NADH (0.15 μ mol), between 0.5 and 1 μ g of pyruvate kinase and 12 units of lactate dehydrogenase in a total volume of 1.0ml. v is expressed as units (μ mol of NADH oxidized/min)/mg of pyruvate kinase.

C to be determined under conditions where different combinations of putative substrates are kept constant. Fig. 2 shows the result of this test. It is evident that inhibition occurs, with increasing C , in every instance except where the free phosphoenolpyruvate (A) and free ADP (B) concentrations are kept constant. This indicates that A , B and C are the substrates of pig liver pyruvate kinase. Other conclusions are drawn below.

Initial-rate studies

Initial-rate studies in which A , B and C were varied were conducted in order to identify the kinetic constants present in the general equation for a three-substrate enzyme (Dalziel, 1969), i.e.

$$\frac{E_0}{v} = \left[\left(\frac{\phi_{ABC}}{C} + \phi_{AB} \right) \frac{1}{B} + \frac{\phi_{AC}}{C} + \phi_A \right] \frac{1}{A} + \left[\left(\frac{\phi_{BC}}{C} + \phi_B \right) \frac{1}{B} + \frac{\phi_C}{C} + \phi_0 \right] \quad (1)$$

Eqn. (1) is cast in a form illustrating that the double-reciprocal plot is linear and also that the eight kinetic constants may be obtained by a stepwise procedure in which the slopes and intercepts of the primary plots are replotted against B^{-1} to obtain secondary line constants, which are then plotted against C^{-1} (Dalziel, 1969).

Fig. 3 shows the fit to eqn. (1) of the initial velocities in the absence of product; together with the linear

Fig. 2. Dependence of the velocity of the reaction catalysed by pig liver pyruvate kinase on increasing Mg^{2+} concentrations

Reaction mixtures contained tetrapropylammonium cacodylate buffer, pH 6.2 (100 μ mol), KCl (100 μ mol), fructose diphosphate (0.20 μ mol), NADH (0.15 μ mol) and 12 units of lactate dehydrogenase in a final volume of 1.0 ml. Phosphoenolpyruvate and ADP concentrations were: (a) and (b) $\bullet$, $\Sigma MgPyr-P$ (0.033 mM) and $\Sigma MgADP$ (0.172 mM); Δ , total $Pyr-P$ (0.1 mM) and total ADP (0.2 mM); $\circ$, $\Sigma Pyr-P$ (0.067 mM) and $\Sigma MgADP$ (0.172 mM); $\blacksquare$, $\Sigma MgPyr-P$ (0.033 mM) and ΣADP (0.028 mM); $\blacktriangle$, $\Sigma Pyr-P$ (0.066 mM) and ΣADP (0.028 mM). Δ is the coincident point for $\bullet$, Δ , $\circ$ and $\blacksquare$ at a free Mg^{2+} concentration of 10 mM. In this and all subsequent Figures, pyruvate kinase was used at a concentration between 0.5 and 2 μ g/ml, the change in [NADH] being recorded spectrophotometrically with a full-scale deflection of 0.1 absorbance unit. The initial rate v is expressed as μ mol of NADH oxidized/min per μ g of pyruvate kinase.

relation in Figs. 4 and 2, they demonstrate that the mechanism is linear in A , B and C and non-sequential (Ping-Pong; Cleland, 1963a) in type. Fig. 5 shows four

Fig. 3. Effect of $[\Sigma ADP]$ on the initial rate of the forward reaction catalysed by pig liver pyruvate kinase with $\Sigma Pyr-P$ as the varied substrate

Assay conditions were as described in Fig. 2. The free Mg^{2+} concentration was 10.0 mM. The concentrations of ΣADP were: $\bullet$, 0.017 mM; $\circ$, 0.025 mM; $\blacksquare$, 0.033 mM; ∇ , 0.067 mM; $\blacktriangle$, 0.125 mM.

Fig. 4. Effect of $[\Sigma Pyr-P]$ on the initial rate of the forward reaction catalysed by pig liver pyruvate kinase with ΣADP as the varied substrate

Assay conditions were as described in Fig. 2. The free Mg^{2+} concentration was 2.0 mM. The concentrations of $\Sigma Pyr-P$ were: $\bullet$, 6.6 μ M; $\blacktriangle$, 10 μ M; $\circ$, 12.8 μ M; Δ , 25 μ M; $\blacksquare$, 66 μ M.

Fig. 5. Plots of the relationships (a)–(c) in which the right-hand sides of the equalities were estimated by the NONSEQUEN program applied to data of the type shown in Figs. 3 and 4 at five free Mg^{2+} concentrations

(a) $\phi_C/[Mg^{2+}] + \phi_0 = I_A I_B$; (b) $\phi_{BC}/[Mg^{2+}] + \phi_B = I_A S_B$; (c) $\phi_{AC}/[Mg^{2+}] + \phi_A = S_A I_B$ where S and I refer to slope and intercept. ●, Pyruvate kinase preparation 1 (specific activity 70 units/mg); ○, pyruvate kinase preparation 2 (specific activity 140 units/mg). Pyruvate kinase preparation 2 was used to obtain the data recorded in Fig. 4 and in Figs. 6–9. In these Figures the ordinate has been multiplied by 2 to correct the data to that of preparation 1.

tertiary replots against C^{-1} in which the data points represent the values of slopes and intercepts of secondary plots against B^{-1} obtained by computation. The evident absence of ϕ_{ABC} confirms the non-sequential nature of the mechanism (Dalziel, 1969), and the presence of ϕ_C demonstrates that the mechanism cannot require the addition of Mg^{2+} to the active site in substrate-bound form.

The test of Macfarlane & Ainsworth (1972) referred to above provides values of i in the relationship

$$v^{-1} = \sum x_i C_i^i$$

determined with different combinations of hypothetical substrates kept constant. Fig. 2 shows that the maximum value of i is +1 when either α and β

or A_i and B_i are kept constant, and indicates therefore that ϕ_{AB} is absent, a conclusion consistent with the triple replot data given in Fig. 5. Fig. 5 shows that a reliable estimate of ϕ_B cannot be obtained. However, as we have shown (Macfarlane & Ainsworth, 1972; Ainsworth & Macfarlane, 1973), inhibition with increasing C is not expected at constant A and β unless ϕ_B is real; a value for ϕ_B may therefore be determined from the limiting slope ($i = +1$) of the relationship of v^{-1} to C obtained when A and β are kept constant.

The possibility exists that the parallel lines evident in Fig. 3 (and which suggest a non-sequential

Fig. 6. Effect of $[\Sigma\text{ADP}]$ on the initial rate of the forward reaction catalysed by pig liver pyruvate kinase at a non-saturating concentration of K^+ and with $\Sigma\text{Pyr-P}$ as the varied substrate

Assay conditions were as in Fig. 2 except that the concentration of free K^+ was 5.0 mM. The concentration of free Mg^{2+} was 10 mM. The concentrations of ΣADP were: ●, 0.015 mM; □, 0.02 mM; ▲, 0.04 mM; ○, 0.066 mM; ■, 0.1 mM.

mechanism) might arise because a substrate, present at a saturating concentration, lies in an ordered sequence between the varied (A) and non-varied (B) substrate (Cleland, 1970; Macfarlane & Ainsworth, 1972). If K^+ ions are regarded as a substrate, in the same sense as are Mg^{2+} ions, then K^+ becomes a candidate for such a position. An experiment was therefore conducted in which the K^+ concentration present in the assay was lowered from 100 to 5 mM.

Fig. 7. Effect of $[\Sigma\text{ADP}]$ on the initial rate of the forward reaction catalysed by pig liver pyruvate kinase in the presence of a fixed concentration of MgATP and with $\Sigma\text{Pyr-P}$ as the varied substrate

Assay conditions were as described in Fig. 2. The concentration of free Mg^{2+} and MgATP were 10.0 and 1.0 mM respectively. The concentrations of ΣADP were: ●, 0.017 mM; □, 0.025 mM; ▲, 0.033 mM; ○, 0.066 mM; ■, 0.1 mM.

Table 1. Constants for the uninhibited reaction catalysed by pig liver pyruvate kinase

Kinetic constants were calculated by assuming that pyruvate kinase has a molecular weight of 200000 (Hess & Kutzbach, 1971). One active site is assumed because the correct number of active sites/pyruvate kinase molecule is unknown.

Kinetic constants		Michaelis constants and maximum velocity	
ϕ_A	$2.5(\pm 0.8) \times 10^{-9} \text{ M} \cdot \text{min}$	K_A	$3.7 \times 10^{-5} \text{ M}$
ϕ_B	$-5.2(\pm 4.8) \times 10^{-9} \text{ M} \cdot \text{min}$	K_B	$5.74 \times 10^{-5} \text{ M}$
	$3.87(\pm 1.2) \times 10^{-9} \text{ M} \cdot \text{min}$		
	(Fig. 2)		
ϕ_C	$2.5(\pm 0.2) \times 10^{-7} \text{ M} \cdot \text{min}$	K_C	$3.72 \times 10^{-3} \text{ M}$
ϕ_{AC}	$3.75(\pm 0.5) \times 10^{-11} \text{ M}^2 \cdot \text{min}$		
ϕ_{BC}	$2.05(\pm 0.3) \times 10^{-11} \text{ M}^2 \cdot \text{min}$	$V_F = 1/\phi_0$	$1.48 \times 10^4 \text{ min}^{-1}$ (catalytic constant)
ϕ_0	$6.75(\pm 1.2) \times 10^{-5} \text{ min}$		or $2.46 \times 10^2 \text{ s}^{-1}$

The data (Fig. 6) again show the features of a non-sequential mechanism and suggest that a substituted enzyme species must be produced during the catalytic cycle.

Another feature of non-sequential mechanisms is that the parallel lines, observed on double-reciprocal plots, become convergent in the presence of a product, and intersect on the vertical axis if the added product competes with the varied substrate (Cleland, 1970). Fig. 7 shows that MgATP causes convergence, and that it must act as a non-competitive inhibitor of the enzyme when phosphoenolpyruvate is the varied substrate.

Values of the kinetic constants obtained by computer analysis of the data are given in Table 1.

Product-inhibition studies

Figs. 8, 9 and 10 show that MgATP, added to the reactant solutions, acts as a non-competitive inhibitor when phosphoenolpyruvate, ADP or Mg^{2+} ion, respectively, is the varied substrate. Fig. 10 also shows that the linear double-reciprocal relationship of initial rate to Mg^{2+} concentration becomes non-linear in the presence of MgATP. An attempt was made to study inhibition by pyruvate by using the assay of Macfarlane & Ainsworth (1972); this failed because 3-phosphoglycerate was found to inhibit pig liver pyruvate kinase. Coupling through

hexokinase and glucose 6-phosphate dehydrogenase also failed because NADP inhibits pyruvate kinase.

Apparent MgATP inhibition constants, obtained directly from the NONCOMP program, are given as (secondary intercept)/(secondary slope). Values of these apparent constants for Figs. 8 and 9 are recorded in Table 2.

Enzyme mechanism

The qualitative features shown in the Results section which bear on the mechanism of pig liver pyruvate kinase may be identified as follows.

(1) The linearity of the double-reciprocal plots indicates either that the enzyme-containing intermediates arising in the catalytic cycle form an unbranched sequence or that an equilibrium random-order mechanism applies.

(2) The test proposed by Macfarlane & Ainsworth (1972), embodied in Fig. 2, shows that free phosphoenolpyruvate, ADP and Mg^{2+} are the substrates of pig liver pyruvate kinase. It may also be concluded that Mg^{2+} is not a product inhibitor and that it must dissociate as part of the nucleotide complex MgATP. Fig. 2 further shows that magnesium-bound phosphoenolpyruvate (α) and ADP (β), the concentrations of which increase with $[Mg^{2+}]$, are not

Fig. 8. Inhibition by MgATP of the forward reaction catalysed by pig liver pyruvate kinase, with Σ Pyr-P as the varied substrate

Assay conditions were as described in Fig. 2. The concentrations of Mg^{2+} and Σ ADP were 10.0 and 0.021 mM respectively. The concentrations of MgATP were: ●, 1.0 mM; △, 0.75 mM; ■, 0.5 mM; ○, 0.25 mM; ▲, 0 mM.

Fig. 9. Inhibition by MgATP of the forward reaction catalysed by pig liver pyruvate kinase, with Σ ADP as the varied substrate

Assay conditions were as described in Fig. 2. The concentrations of Mg^{2+} and Σ Pyr-P were 10.0 and 0.24 mM respectively. The concentrations of MgATP were: ●, 1.0 mM; △, 0.75 mM; ■, 0.5 mM; ○, 0.25 mM; ▲, 0 mM.

Fig. 10. Inhibition by MgATP of the forward reaction catalysed by pig liver pyruvate kinase with Mg^{2+} as the varied substrate

Assay conditions were as described in Fig. 2. The concentrations of ΣADP and $\Sigma Pyr-P$ were 0.028 and 0.08 mM respectively. The concentrations of MgATP were: ■, 2.0 mM; ▼, 1.0 mM; ▲, 0.5 mM; ●, 0 mM.

Table 2. Inhibition constants arising from the inhibition of pig liver pyruvate kinase by MgATP

Apparent inhibition constants obtained directly from the NONCOMP program are given as (secondary intercept)/(secondary slope).

Varied substrate	Fixed substrate (mM)	Replot	Apparent inhibition constant (mM)
A	B:0.021	Slope	0.52
		Intercept	4.0
B	A:0.24	Slope	1.34
		Intercept	1.37

inhibitors of the reaction (Macfarlane & Ainsworth, 1972).

(3) The parallel lines observed on double-reciprocal plots when phosphoenolpyruvate is the varied and ADP is the non-varied substrate indicate that the binding of these two substrates by the enzyme must be separated by a step in which a product is released. In turn, this suggests that a random-order mechanism does not apply.

The non-sequential nature of the mechanism therefore suggests that phosphoenolpyruvate is bound by the enzyme before pyruvate is released and that both events precede the ordered binding of ADP and release of MgATP.

(4) The tertiary replots, shown in Fig. 5, demonstrate the presence of kinetic constants ϕ_{AC} and ϕ_{BC} in the rate equation for pyruvate kinase. Dalziel (1969) has shown that ϕ_{AC} requires that A and C bind sequentially to the enzyme and similarly that ϕ_{BC} requires the sequential binding of B and C. These requirements, together with the need to separate the binding of A and B by the release of a product (point 3), therefore suggest that not one but two Mg^{2+} ions bind to the enzyme during its catalytic cycle.

(5) The failure to observe product inhibition by Mg^{2+} has already led to the conclusion that one Mg^{2+} ion dissociates as part of the nucleotide complex MgATP (point 2). The presence of a second Mg^{2+} ion in the catalytic cycle, indicated by point 4, therefore requires a further device to prevent the reappearance of Mg^{2+} inhibition. We suggest, accordingly, that the enzyme bears only one Mg^{2+} ion at a time and that the binding of one Mg^{2+} ion is competitive with the binding of the other.

(6) The observation that MgATP acts as a non-competitive inhibitor when phosphoenolpyruvate and Mg^{2+} is the varied substrate suggests that an isomerization of the enzyme is interposed between the binding of these substrates and MgATP.

The preceding arguments suggest that either of Mechanisms 1 or 2 might describe the catalytic role of pig liver pyruvate kinase.

In these mechanisms, E stands for the enzyme-fructose 1,6-diphosphate- K^+ complex, and F stands

for an isomerized complex of E formed during one of the steps preceding the release of Q. Q represents the Mg^{2+} complexed forms of ATP, and P, pyruvate. The concentration dependence of each step is indicated.

The distribution of enzyme intermediates in Mechanisms 1 and 2 have been derived by King & Altman's (1956) method, for the forward reaction in the presence and absence of products added singly. The concentration terms that arise (there are no constant terms) together with the ϕ terms that derive from them are as follows:

For Mechanism 1: $ABC^2 + ABC^2P + ABC^2Q (\phi_{AB}^{PQ})$; $ABC + ABCP (\phi_B^P)$; $C^2A + C^2AQ + C^2AP (\phi_B^{PQ})$; $C^2B + C^2BP (\phi_B^P)$; $AC + ACQ (\phi_{BC}^Q)$; $CB + CBQ + CBP (\phi_{AC}^{PQ})$; $CP + CQ (\phi_{AB}^{PQ})$; $C^2P (\phi_{AB}^{PQ})$; $AQ (\phi_{BC}^{PQ})$; $Q (\phi_{ABC}^{PQ})$.

For Mechanism 2: $ABC^2 + ABC^2P + ABC^2Q (\phi_{AB}^{PQ})$; $ABC + ABCP (\phi_B^P)$; $C^2A + C^2AQ + C^2AP (\phi_B^{PQ})$; $C^2B + C^2BQ (\phi_B^Q)$; $AC + ACP + ACQ (\phi_{BC}^{PQ})$; $CB + CBQ + CBP (\phi_{AC}^{PQ})$; $CP + CQ (\phi_{AB}^{PQ})$; $C^2Q (\phi_{AB}^{PQ})$; $Q (\phi_{ABC}^{PQ})$.

In these lists, Dalziel's (1969) nomenclature for the kinetic constants has been modified to indicate which inhibitor, added singly to the reaction solution, affects their value, i.e. ϕ_C^P stands for $\phi_C(1+P/K)$ where K is a ratio of rate constants. In addition, the nomenclature has been extended further to show kinetic constants for substrates that are only present when a given inhibitor is also present, i.e. ϕ_{AB}^{PQ} . For both mechanisms, the numerator of the rate equation is a term in ABC^2 .

The inverted rate equations are given below as a function of A^{-1} in the presence or absence of Q:

$$\frac{E_0}{v} = \left[\left(\frac{ABC^2\phi^Q}{C^2} + \frac{ABC\phi^Q}{C} \right) \frac{1}{B} + \frac{\phi_{AC}^Q}{C} + \phi_A \right] \frac{1}{A} + \left[\left(\frac{BC^2\phi^Q}{C^2} + \frac{\phi_{BC}^Q}{C} + \phi_B^Q \right) \frac{1}{B} + \frac{\phi_C^Q}{C} + \phi_B^Q \right] \quad \text{Mechanism 1}$$

and

$$\frac{E_0}{v} = \left[\left(\frac{ABC^2\phi^Q}{C^2} + \frac{ABC\phi^Q}{C} + \phi_{AB}^{PQ} \right) \frac{1}{B} + \frac{\phi_{AC}^Q}{C} + \phi_A^Q \right] \frac{1}{A} + \left[\left(\frac{\phi_{BC}^Q}{C} + \phi_B^Q \right) \frac{1}{B} + \frac{\phi_C^Q}{C} + \phi_B^Q \right] \quad \text{Mechanism 2}$$

The equations may also be written as a function of C^{-1} :

$$\frac{E_0}{v} = \left[\left(\frac{ABC^2\phi^Q}{AB} + \frac{BC^2\phi^Q}{B} \right) \frac{1}{C} + \frac{ABC\phi^Q}{AB} + \frac{\phi_{AC}^Q}{A} + \frac{\phi_{BC}^Q}{B} + \phi_C^Q \right] \frac{1}{C} + \left(\frac{\phi_A}{A} + \frac{\phi_B^Q}{B} + \phi_B^Q \right) \quad \text{Mechanism 1}$$

and

$$\frac{E_0}{v} = \left(\frac{ABC^2\phi^Q}{ABC} + \frac{ABC\phi^Q}{AB} + \frac{\phi_{AC}^Q}{A} + \phi_{BC}^Q + \phi_C \right) \frac{1}{C} + \left(\frac{AB\phi^Q}{AB} + \frac{\phi_A^Q}{A} + \frac{\phi_B^Q}{B} + \phi_B^Q \right) \quad \text{Mechanism 2}$$

Both equations are consistent with the experimental data that have been presented. One point deserves emphasis. Both mechanisms predict a linear relationship between v^{-1} and the reciprocals of the substrate concentrations, A^{-1} and B^{-1} , when varied independently in the presence or absence of Q: note particularly, however, that a linear relation between v^{-1} and C^{-1} is only predicted in the absence of Q, in the presence of Q a non-linear relation is expected. The presence of this feature in Fig. 10 offers additional qualitative support for the hypothesis that two Mg^{2+} ions are involved in the catalytic cycle.

We do not have evidence at this time to decide which of the two mechanisms more closely describes the kinetic behaviour observed. However, the second mechanism, in which phosphoenolpyruvate is bound first to the enzyme, may be preferred because of its similarity to the mechanisms of the yeast (Macfarlane & Ainsworth, 1972) and human liver enzymes (Balinsky *et al.*, 1972). Even so, this mechanism is probably incomplete; for example, the absence of pyruvate-inhibition data prevents us determining whether dead-end inhibition by pyruvate occurs as in the yeast and rabbit muscle enzymes (Ainsworth & Macfarlane, 1973).

Discussion

In discussing the events at the active sites of yeast and rabbit muscle pyruvate kinases we suggested that bound Mg^{2+} acts as a bridge between the phosphate group of phosphoenolpyruvate and the terminal phosphate group of ADP, assisting the phosphorylation of the latter and ultimately being eliminated between the β - and γ -phosphate groups of ATP. A very similar sequence of events may be envisaged for the pig liver enzyme, in which the first Mg^{2+} ion that is bound assists in the transfer of the phosphate group of phosphoenolpyruvate to a receptor group on the protein. After the transfer is complete, the Mg^{2+} ion dissociates from the protein. The subsequent transfer of the phosphate group from its point of attachment with the protein to ADP again requires the assistance of Mg^{2+} , but bound now to a second site on the enzyme. We further postulate that the two Mg^{2+} -binding sites cannot be occupied simultaneously, so that their binding is kinetically competitive (Monod *et al.*, 1963). The physical cause of this behaviour may well be the conformational change suggested by our results,

triggered directly by Mg^{2+} binding or indirectly by the substitution/release of the transferred phosphate residue at the receptor group of the enzyme.

The kinetic mechanism for pig liver pyruvate kinase resembles that of the enzyme from *Brevibacterium flavium* (Osaki & Shiio, 1969) in which there is also non-sequential binding of phosphoenolpyruvate and ADP and in which ADP and ATP, though a like substrate-product pair, are non-competitive. It is noteworthy that the kinetic mechanism found for the pig liver enzyme resembles that for the *Brevibacterium* enzyme but differs from the ordered sequential mechanisms found for baker's yeast and human liver L-type pyruvate kinase (Macfarlane & Ainsworth, 1972; Balinsky *et al.*, 1972). One general feature emerges, however, in that all these L-type pyruvate kinases exhibit mechanisms in which phosphoenolpyruvate binds to the enzyme before ADP with pyruvate released before MgATP. Ainsworth & Macfarlane (1973) concluded from the asymmetric order of substrate addition (phosphoenolpyruvate, ADP, Mg^{2+}) and product release (pyruvate, MgATP) found for yeast pyruvate kinase that the locus within the active site where conformational change is triggered lies in the region of overlap between the binding sites of phosphoenolpyruvate and MgATP. Though pyruvate kinase from baker's yeast, rabbit muscle and pig liver have different kinetic mechanisms the Michaelis constants for ADP and phosphoenolpyruvate are very similar (Macfarlane & Ainsworth, 1972; Ainsworth & Macfarlane 1973) and there is only a threefold variation in the Michaelis constant for Mg^{2+} .

The concentration of fructose diphosphate in liver fluctuates during starvation and feeding between 5 and $20\mu M$ (Koster *et al.*, 1972). Hess & Kutzbach (1971) showed that the major pyruvate kinase species in crude pig liver homogenates is an enzyme-fructose diphosphate complex and that the concentration of free pyruvate kinase increases in proportion during purification. Hess & Kutzbach (1971) also demonstrated that the purified enzyme can be converted into an enzyme-fructose diphosphate complex on incubation with fructose diphosphate for 24h. Our results show that the further addition of 0.2mM-fructose diphosphate to this complex before the reaction commences has no

effect on the rate. With phosphoenolpyruvate as the varied substrate, the lines of a Lineweaver-Burk plot, at several fixed ADP concentrations and 10mM free Mg^{2+} , obtained by using enzyme incubated for 24h with fructose diphosphate, are parallel, suggesting a non-sequential mechanism. The apparent Michaelis constants for ADP and phosphoenolpyruvate obtained from these data were $1.37 \times 10^{-4} M$ and $5.57 \times 10^{-5} M$ respectively and may be compared with values of $1.55 \times 10^{-4} M$ and $6.1 \times 10^{-5} M$ for ADP and phosphoenolpyruvate obtained under identical conditions by using enzyme which had not previously been incubated with fructose diphosphate.

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