A Steady-State Kinetic Analysis of the Fructose 1,6-Bisphosphate-Activated Pyruvate Kinase from *Carcinus maenas* Hepatopancreas

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1. An investigation of the reaction mechanism of the fructose 1,6-bisphosphate-activated pyruvate kinase isolated from the hepatopancreas of the crab *Carcinus maenas* was conducted. The enzyme was assayed in the presence of 500 μM -fructose 1,6-bisphosphate, 75 mM-KCl and 8 mM- $\text{Mg}^{2+}_{\text{free}}$ at 25°C. The results are consistent with a rapid-equilibrium random mechanism. 2. Evidence is presented that suggests the formation of two mixed-substrate-product dead-end complexes, enzyme-ADP-pyruvate and enzyme-ADP-ATP. 3. Competitive substrate inhibition was observed for both substrates, ADP and phosphoenolpyruvate, suggesting the formation of the complexes enzyme-ADP-ADP and enzyme-phosphoenolpyruvate-phosphoenolpyruvate in the suggested mechanism. 4. Data from the ATP product-inhibition studies indicate the formation of the complex enzyme-ATP-ATP. This suggests that in the reverse reaction ATP also will show substrate inhibition. 5. The presence of a saturating concentration of fructose 1,6-bisphosphate does not cause full activation of the purified preparations of the enzyme. 6. Pyruvate kinase activity in the supernatant of a hepatopancreas homogenate was completely activated by fructose 1,6-bisphosphate, suggesting that the binding of this ligand to the purified pyruvate kinase was impaired.

Pyruvate kinase (EC 2.7.1.40) is an important enzyme of the glycolytic pathway. The enzyme has been purified from a variety of sources, and, basically, two types of activity exist. One type is allosterically activatable by the glycolytic intermediate fructose 1,6-bisphosphate, whereas the other is insensitive to this compound. The two types of activity have been designated type L and type M respectively (Tanaka *et al.*, 1967). A further characteristic of the type-L pyruvate kinases is that they exhibit sigmoidal saturation kinetics with the substrate phosphoenolpyruvate in the absence of fructose 1,6-bisphosphate. In the presence of this allosteric activator normal hyperbolic saturation kinetics are observed.

Steady-state kinetic analyses of the fructose 1,6-bisphosphate-activated type-L pyruvate kinase isolated from either yeast or pig liver have suggested that the former catalyses an ordered mechanism (Macfarlane & Ainsworth, 1972), whereas the latter catalyses a Ping Pong mechanism (Macfarlane & Ainsworth, 1974). Isotope-exchange studies of the pig liver enzyme, however, are not in agreement with this latter conclusion and indicate that the enzyme catalyses a sequential mechanism (Giles *et al.*, 1976a). Subsequent studies using a less-pure enzyme

from rabbit liver have supported this conclusion (Dann & Britton, 1977).

The type-M pyruvate kinase from rabbit skeletal muscle has been reported to catalyse a rapid-equilibrium random mechanism (Reynard *et al.*, 1961; Mildvan & Cohn, 1966; Ainsworth & Macfarlane, 1973), although isotope-exchange studies have suggested that the rapid-equilibrium assumption is only an approximation to the true situation (Robinson & Rose, 1972; Giles *et al.*, 1976c; Dann & Britton, 1978). In addition this enzyme has been reported to form the dead-end complexes enzyme-ADP-ADP (Janson & Cleland, 1974) and enzyme-ATP-ATP (Giles *et al.*, 1976c). No such abortive complexes have been reported for the type-L pyruvate kinase.

The pyruvate kinase from the hepatopancreas of the crustacean *Carcinus maenas* has been purified (Giles *et al.*, 1976b) and shown to be a type-L enzyme (Giles *et al.*, 1977). The present paper reports an investigation of the reaction mechanism of this enzyme by the use of steady-state kinetics, looking in particular for the existence of any dead-end complexes that may form during the catalytic cycle. All reactions took place at pH 7.4 and 25°C; the enzyme was fully activated by the addition of 500 μM -fructose 1,6-bisphosphate, K^+ was present at

all times at 75 mM and the free Mg^{2+} concentration was held constant at 8 mM. All references to the concentration of substrates and products are in terms of their total chemical concentrations.

Theory

The experimental data obtained in the presence of a constant concentration of free Mg^{2+} are consistent with a rapid-equilibrium random Bi-Bi-type mechanism with five dead-end complexes. The reaction pathway is illustrated in Scheme 1. The full rate equation for the mechanism is given by:

$$v = \frac{V_1 AB - V_2 \left(\frac{K_{1a} K_b}{K_{1p} K_q} \right) PQ}{K_{1a} K_b + K_b A + K_a B + AB + \frac{K_{1a} K_b P}{K_{1p}} + \frac{K_{1a} K_b Q}{K_{1q}} + \frac{K_{1a} K_b PQ}{K_{1p} K_q} + \frac{K_b A^2}{K_{1a}} + \frac{K_a B^2}{K_{1b}} + \frac{K_{1a} K_b Q^2}{K_{1q} K_{1q}} + \frac{K_b A Q}{K_{1q}^d} + \frac{K_b A P}{K_{1p}^d}} \quad (1)$$

where A , B , P and Q refer to the total concentrations of ADP, phosphoenolpyruvate, pyruvate and ATP respectively, and V_1 and V_2 are the maximum velocities in the forward and reverse reactions. The nomenclature of Cleland (1963) is used for the kinetic constants with the addition that constants of the form K_1 indicate formation of an enzyme species that is not on the productive catalytic pathway. The kinetic constants are defined in Scheme 1.

Initial-rate data

In the absence of products the full rate equation (1) simplifies to:

$$v = \frac{VAB}{K_{1a} K_b + K_a B + K_b A + AB + \frac{K_b A^2}{K_{1a}} + \frac{K_a B^2}{K_{1b}}} \quad (2)$$

On inverting, one can obtain two equations (3 and 4) that show the variation in reciprocal rate as a function of either $1/A$ or $1/B$ with the second non-variable substrate held at a fixed concentration.

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_b}{B} \right) + \frac{K_a}{V} \left(1 + \frac{K_{1a} K_b}{K_a B} + \frac{K_b A^2}{K_a K_{1a} B} + \frac{B}{K_{1b}} \right) \frac{1}{A} \quad (3)$$

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_a}{A} \right) + \frac{K_b}{V} \left(1 + \frac{K_{1a}}{A} + \frac{K_a B^2}{K_b K_{1b} A} + \frac{A}{K_{1a}} \right) \frac{1}{B} \quad (4)$$

Scheme 1. Reaction scheme for the rapid-equilibrium random Bi-Bi mechanism with five dead-end complexes proposed for the fructose 1,6-bisphosphate-activated pyruvate kinase from *C. maenas* hepatopancreas

The nomenclature used is based on that of Cleland (1963). A , B , P and Q refer to the reactants ADP, phosphoenolpyruvate, pyruvate and ATP respectively. k_1 and k_2 are the rate-limiting first-order rate constants for the inter-conversion of the ternary complexes in the forward and reverse reactions respectively.

In the graphs presented below the initial velocity of the reaction was determined at total concentrations of the variable substrate such that substrate inhibition was not apparent. That is the contribution of the A^2 term in eqn. (3) and of the B^2 term, in eqn. (4) are negligible. This is the asymptotic condition whereby substrate inhibition by the variable substrate is not significantly affecting the initial rate of the reaction. In double competitive substrate inhibition one would expect to find, for both primary plots, a linear relationship between the intercepts of the asymptotes in the primary plot and the reciprocal of the concentration of the second substrate and a hyperbolic relationship (similar to that for substrate inhibition) between the slope of the asymptotes of the primary plots and the reciprocal of the concentration of the second substrate.

Product inhibition studies

Pyruvate against ADP. Eqn. (5) is the reciprocal form of the rate equation and can be seen to indicate linear non-competitive inhibition if the asymptotic condition applies (i.e. no ADP substrate inhibition is manifest).

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_b}{B} + \frac{K_b P}{K_{ip}^d B} \right) + \frac{K_a}{V} \left[1 + \frac{B}{K_{ib}} + \frac{K_b A^2}{K_a K_{ia} B} + \frac{K_{ia} K_b}{K_a B} \left(1 + \frac{P}{K_{ip}} \right) \right] \frac{1}{A} \quad (5)$$

Pyruvate against phosphoenolpyruvate. Eqn. (6) represents linear competitive inhibition for the asymptotic condition. Note that the slope of the slope replot contains the term $(K_b/V)[(K_{ia}/K_{ip}A) + (1/K_{ip}^d)]$ and hence separate values for K_{ip} and K_{ip}^d cannot be calculated from these data.

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_a}{A} \right) + \frac{K_b}{V} \left[1 + \frac{K_{ia}}{A} \left(1 + \frac{P}{K_{ip}} \right) + \frac{A}{K_{ia}} + \frac{K_a B^2}{K_b K_{ib} A} + \frac{P}{K_{ip}^d} \right] \frac{1}{B} \quad (6)$$

ATP against ADP. Eqn. (7) represents non-competitive inhibition in which the intercepts are a linear function, and the slopes a parabolic function, of ATP concentration, provided that the asymptotic condition applies for both substrates.

$$\frac{1}{v} = \frac{1}{V} \left[1 + \frac{K_b}{B} \left(1 + \frac{Q}{K_{iq}^d} \right) \right] + \frac{K_a}{V} \times \left[1 + \frac{B}{K_{ib}} + \frac{K_b A^2}{K_a K_{ia} B} + \frac{K_{ia} K_b}{K_a B} \times \left[1 + \frac{Q}{K_{iq}} \left(1 + \frac{Q}{K_{iq}} \right) \right] \right] \frac{1}{A} \quad (7)$$

ATP against phosphoenolpyruvate. Eqn. (8) represents parabolic competitive inhibition (i.e. the slopes are a parabolic function of ATP concentration), provided that the asymptotic condition applies for both ADP and phosphoenolpyruvate. Note that the linear term of the slope replot contains the term $(K_b/V)(K_{ia}/K_{iq}A) + (1/K_{iq}^d)$ and hence values for K_{iq} and K_{iq}^d cannot be calculated from this value.

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_a}{A} \right) + \frac{K_b}{V} \left[1 + \frac{A}{K_{ia}} + \frac{K_a B^2}{K_b K_{ib} A} + \frac{K_{ia}}{A} \times \left[1 + \frac{Q}{K_{iq}} \left(1 + \frac{Q}{K_{iq}} \right) \right] + \frac{Q}{K_{iq}^d} \right] \frac{1}{B} \quad (8)$$

Data analysis

The initial-rate data were fitted to the Michaelis-Menten equation (9) by the unweighted hyperbolic least-squares method of Wilkinson (1961) after a graphical check that the double-reciprocal plots of reaction rate against substrate concentration were linear. Slope and ordinate intercept secondary replots, including the s.e. of the variables, were constructed and used to identify the form of the rate equation. The lines in the primary plots shown in the text correspond to the least-squares fit of each line to eqn. (9). When substrate inhibition was apparent in a line the data were fitted to the relevant rate eqn. (10), and the slope and ordinate intercept of the asymptote calculated. The values obtained were not significantly different from those obtained using a restricted range of substrate concentrations (to avoid the inhibition) and fitting to eqn. (9). Finally all the data obtained in the absence of products were fitted directly to eqn. (2).

In the product-inhibition experiments a similar strategy was followed. The data were fitted directly to eqn. (9), and the secondary replots of either the ordinate intercept or the slope of the primary plot as a function of inhibitor concentration were constructed. These secondary replots were used to determine the form of the rate equation. In the case of ATP inhibition against ADP the data were fitted directly to eqn. (11) (where $D = K_{ia}K_b + K_aB + K_bA + AB + K_bA^2/K_{ia} + K_aB^2/K_{ib}$, and the values determined in the initial-rate studies were substituted). For the other inhibition studies the data were fitted directly to the general rate equation for competitive inhibition, parabolic competitive inhibition or non-competitive inhibition as appropriate (Cleland, 1967).

$$v = \frac{VS}{K_m + S} \quad (9)$$

$$v = \frac{VS}{K_m + S + S^2/K_i} \quad (10)$$

$$v = \frac{VAB}{D + \text{coeff. } P(P) + \text{coeff. } AP(AP) + \text{coeff. } P^2(P^2)} \quad (11)$$

All computer fittings were based on the method of Cleland (1967) and were run on a Digital Corp. PDP-12 computer with the use of programs written locally in Focal-12.

Experimental

Enzymes and substrates

Pyruvate kinase from the hepatopancreas of *Carcinus maenas* was prepared as described by Giles *et al.* (1976b) in their preparation B. The enzyme had a specific activity of 240 μmol of pyruvate produced/min per mg of protein at 25°C.

Pyruvate kinase, aldolase (EC 4.1.2.13) and triose phosphate isomerase (EC 5.3.1.1) from rabbit muscle and the yeast enzymes, hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Sigma Chemical Co., London S.W.6, U.K., as were sodium pyruvate and Trizma base. Pig heart lactate dehydrogenase (EC 1.1.1.27), ADP, ATP, fructose 1,6-bisphosphate and phosphoenolpyruvate (tricyclohexylamine salt) were supplied by Boehringer Corp., London W.5, U.K.

All other chemicals were supplied by BDH Chemicals, Poole, Dorset, U.K.

Methods

Standard kinetic assay of pyruvate kinase. In order to compare the rates of reaction obtained in experiments on different days and/or on different batches of enzyme, the activity was normalized by measurement under a standard set of conditions. The reaction mixture used contained 0.15 μmol of NADH, 0.50 μmol of ADP, 0.80 μmol of phosphoenolpyruvate, 8.0 μmol of $\text{Mg}^{2+}_{\text{free}}$ added as MgCl_2 , 1.0 μmol of EGTA, 0.5 μmol of fructose 1,6-bisphosphate, 75.0 μmol of KCl, 25.0 μmol of Tris/HCl (pH 7.4) and 16 units of lactate dehydrogenase in a final volume of 1.0 ml. The reaction was initiated by the addition of pyruvate kinase after the reaction mixture had been equilibrated to 25°C. Oxidation of NADH was followed at 340 nm in a Perkin-Elmer 356 dual-wavelength spectrophotometer, operating in the split-beam mode, connected to a model 165 Perkin-Elmer chart recorder.

Experimental kinetic assay of pyruvate kinase. Initial-rate data and product-inhibition patterns with ATP as the inhibitor were obtained as described above, except that the concentrations of ADP and phosphoenolpyruvate were varied as indicated in the

Figures. When pyruvate was used as the product inhibitor the direct spectrophotometric assay of Pon & Bondar (1967) was used. The reaction mixture was the same as that described for the inhibition by ATP, except that NADH and lactate dehydrogenase were omitted.

Before reaction rates were measured all solutions, except those of the enzymes, were filtered under positive pressure through a 0.45 μm Millipore filter and degassed *in vacuo*.

Solutions of substrates, products and cofactors were prepared daily and their concentrations determined immediately before use. The concentration of ADP was determined with pyruvate kinase and lactate dehydrogenase (Adam, 1963), ATP with hexokinase and glucose 6-phosphate dehydrogenase (Lamprecht & Trautschold, 1963), pyruvate with lactate dehydrogenase (Bücher *et al.*, 1963) and phosphoenolpyruvate with pyruvate kinase and lactate dehydrogenase (Czok & Eckert, 1963). In addition, ATP, which was purified as previously described (Giles *et al.*, 1976c), and ADP were assayed for cross-contamination with each other and this was allowed for in all the relevant calculations.

The $\text{Mg}^{2+}_{\text{free}}$ concentration was obtained by calculation, allowing for its chelation with ADP, ATP, phosphoenolpyruvate, fructose 1,6-bisphosphate and EGTA by using dissociation constants based on those given by Macfarlane & Ainsworth (1972) and Sillén & Martell (1971). Calculations were accomplished with the aid of a Hewlett-Packard 9810 desktop computer by using a modified Fibonacci search procedure.

The dissociation constants used were calculated to be those applicable at 25°C in a medium of *I* 0.2. In the current series of experiments no attempt was made to maintain the ionic strength constant. The maximum variation in ionic strength occurred between the assays at low substrate concentrations in the absence of products and those at high substrate concentrations in the presence of 15 mM-ATP. Calculation of the *pK* for MgADP^- dissociation shows that it varied from -3.41 to -3.43 between these extreme conditions. This change represents less than a 5% change in the dissociation constant itself. Similar calculations for the other constants employed showed that this was the maximum expected percentage change; in several cases only 1–2% changes occurred.

As a consequence of keeping the free Mg^{2+} concentration constant the concentration of the magnesium-chelate forms of the nucleotides and of phosphoenolpyruvate were always in a constant ratio to that of the magnesium-free forms. In the conditions of constant pH and metal-ion concentrations used 99.2, 93.5 and 42.2% of the ATP, ADP and phosphoenolpyruvate respectively were present as their magnesium-chelates.

Fig. 1. Effect of total ADP concentration on the initial rate of the forward reaction of *C. maenas* pyruvate kinase when phosphoenolpyruvate is the variable substrate. The enzyme was assayed as described in the Experimental section. The initial rate measured, v , is expressed as a ratio to that measured in standard assay conditions, v_k . These standard assay conditions are described in the Experimental section. The total ADP concentrations used were: $\square$, 45.3 μM ; $\blacksquare$, 56.6 μM ; $\circ$, 79.3 μM ; $\bullet$, 113 μM ; $\triangle$, 226 μM ; $\blacktriangle$, 800 μM ; ∇ , 2.4 mM; $\blacktriangledown$, 9.5 mM.

Fig. 2. Effect of phosphoenolpyruvate concentration on the initial rate of the forward reaction of *C. maenas* pyruvate kinase when ADP is the variable substrate. The assay conditions were as described in Fig. 1. The concentrations of total phosphoenolpyruvate used were: $\square$, 32.6 μM ; $\blacksquare$, 65.2 μM ; $\circ$, 196 μM ; $\bullet$, 326 μM ; $\triangle$, 1.09 mM; $\blacktriangle$, 4.57 mM. Two lines at 131 μM - and 545 μM -phosphoenolpyruvate have been omitted for clarity, but were included in the analysis.

Fig. 3. Secondary replots of the data in Figs. 1 and 2 in terms of the ordinate intercepts and slopes of these lines as a function of the reciprocal concentration of the fixed substrate

(a) The intercept replot obtained when ADP was the primary variable substrate, and the linear least-squares regression line of the first seven points. Deviation from this line is seen at low phosphoenolpyruvate concentrations; see the text for an explanation. (b, c) The slope replots obtained when either phosphoenolpyruvate (b) or ADP (c) was the primary variable substrate. The least-squares fit of the data to eqn. (10) is shown. The intercepts are a dimensionless ratio of activities and the slopes have units of mM. The error bars indicate the s.e.m. of the parameters obtained by least-squares fit of each line to eqn. (9).

Results

Initial-rate studies

Initial-rate studies over a wide range of substrate concentrations showed substrate inhibition by both substrates. In order to analyse the type of substrate inhibition occurring (Cleland, 1972) the variable substrate was varied over a restricted range, such that substrate inhibition by it was not manifest. The fixed substrate was varied over a wide concentration range and the effect of inhibition on the slopes and intercepts of the primary plot observed. Fig. 1 is the double-reciprocal plot of the reaction rate against variable total ADP concentration and Fig. 2 is a similar plot between rate and total phosphoenolpyruvate concentration. In both Figures the slopes of the lines decrease as the concentration of the fixed substrate increases, but as the concentration of the fixed substrate increases further a

minimum occurs, after which the slopes start to increase. Secondary replots of the slopes and intercepts against reciprocal concentration of the fixed substrate are shown in Fig. 3(a-c). Both of the intercept replots are linear, whereas the slope replots are characteristically hyperbolic. These patterns are diagnostic of double competitive substrate inhibition and are consistent with eqns. (3) and (4). The kinetic constants obtained by fitting the data to eqn. (2) are summarized in Table 1.

Table 1. Kinetic parameters of the uninhibited pyruvate kinase reaction

The constants were obtained by fitting the experimental data obtained at $8\text{ mM-Mg}^{2+}_{\text{free}}$ and 75 mM-K^{+} to eqn. (2). The constants are quoted in terms of total chemical concentration, not in terms of the substrate species.

K_a	$91.4 \pm 7.4\ \mu\text{M}$
K_b	$30.6 \pm 6.1\ \mu\text{M}$
K_{ia}	$77.4 \pm 21.4\ \mu\text{M}$
K_{ib}	$34.4 \pm 9.5\ \mu\text{M}$
K_{ia}	$3.53 \pm 0.73\ \text{mM}$
K_{ib}	$2.78 \pm 0.43\ \text{mM}$
V	1.21 ± 0.02

Fig. 4. Inhibition by pyruvate of the forward reaction catalysed by *C. maenas* pyruvate kinase with ADP as the variable substrate

The enzyme was assayed by the direct spectrophotometric assay of Pon & Bondar (1967), as described in the Experimental section. The total concentration of phosphoenolpyruvate was held constant at $35.8\ \mu\text{M}$. The concentrations of pyruvate used were: $\blacktriangle$, 7.8 mM ; $\square$, 4.67 mM ; $\blacksquare$, 3.12 mM ; $\circ$, 1.56 mM ; $\bullet$, 0 .

Fig. 5. Inhibition by pyruvate of the forward reaction catalysed by *C. maenas* pyruvate kinase with phosphoenolpyruvate as the variable substrate

The double-reciprocal plot of enzyme activity against phosphoenolpyruvate concentration with a constant total ADP concentration of $104\ \mu\text{M}$. The concentrations of pyruvate used were: $\square$, 4.67 mM ; $\blacksquare$, 3.12 mM ; $\circ$, 1.56 mM ; $\bullet$, 0 .

Fig. 6. Inhibition by ATP of the forward reaction catalysed by *C. maenas* pyruvate kinase at a non-saturating concentration of phosphoenolpyruvate with ADP as the variable substrate

The assay conditions were as described in Fig. 1. The total concentration of phosphoenolpyruvate was held constant at $39.2\ \mu\text{M}$. The total concentrations of ATP used were: ∇ , 15 mM ; $\blacktriangledown$, 9.55 mM ; $\triangle$, 7.51 mM ; $\blacktriangle$, 5.36 mM ; $\square$, 3.82 mM ; $\blacksquare$, 2.44 mM ; $\circ$, 1.22 mM ; $\bullet$, 0 . Two additional lines at 0.153 mM - and 0.61 mM -ATP, and an additional point on the zero-concentration line at $36\ \mu\text{M}$ -ADP, have been omitted for clarity, but were included in the full analysis.

Product-inhibition studies

Figs. 4 and 5 illustrate the inhibition patterns obtained with pyruvate as the product when ADP and phosphoenolpyruvate respectively are the variable substrates. The secondary replot of slopes and intercepts of Fig. 4 against pyruvate concentration show that the inhibition is linear non-competitive. The inhibition by pyruvate when phosphoenolpyruvate is the variable substrate is linear competitive. These inhibition patterns are as predicted by eqns. (5) and (6), provided that the concentrations of the substrate used are small compared with their

respective substrate inhibition constants K_{1a} and K_{1b} . This is the asymptotic condition where substrate inhibition with respect to both substrates is not significantly affecting the initial rate of the reaction.

When ATP was the product and ADP the variable substrate, two families of lines were obtained: one (Fig. 6) at a low ($39.2\mu\text{M}$) and the other (Fig. 7) at a high (2.27mM) concentration of the fixed substrate phosphoenolpyruvate. The ordinate intercepts of both of these Figures are a linear function of total ATP concentration. The slope

Fig. 7. Inhibition by ATP of the forward reaction of *C. maenas* pyruvate kinase at a saturating concentration of phosphoenolpyruvate with ADP as the variable substrate

The assay conditions were as described in Fig. 1. The total concentration of phosphoenolpyruvate was held constant at 2.27mM . The total ATP concentrations used were: $\blacktriangle$, 15.3mM ; $\square$, 9.55mM ; $\blacksquare$, 5.15mM ; $\circ$, 2.57mM ; $\bullet$, 0 . Two additional lines at 0.775mM - and 1.55mM -ATP have been omitted for clarity, but were included in the full analysis.

Fig. 8. Secondary replots of the slopes of the lines in Figs. 6 and 7

The slope replots are shown as a function of total ATP concentration. The phosphoenolpyruvate concentrations used were: $\circ$, 2.27mM ; $\bullet$, $39.2\mu\text{M}$. The two lines at 2.27mM -phosphoenolpyruvate correspond to the linear (—) and parabolic (----) least-squares regression lines. The line at $39.2\mu\text{M}$ -phosphoenolpyruvate is the parabolic least-squares regression line. The error bars indicate the S.E.M. of the parameters obtained from the hyperbolic least-squares regression analysis of each line in Figs. 6 and 7 to eqn. (9).

Fig. 9. Inhibition by ATP of the forward reaction of *C. maenas* pyruvate kinase with phosphoenolpyruvate as the variable substrate

The assay conditions are as described in Fig. 1. (a) The double-reciprocal plot of enzyme activity against phosphoenolpyruvate concentration. The total concentration of ADP was $104\mu\text{M}$. The total ATP concentrations used were: Δ , 5mM ; $\blacktriangle$, 3.06mM ; $\square$, 2.26mM ; $\blacksquare$, 1.22mM ; $\circ$, 0.95mM ; $\bullet$, 0 . (b) Secondary replot of the slopes of the lines in (a) as a function of ATP concentration. The linear (—) and parabolic (----) least-squares regression curves are shown. The error bars indicate the S.E.M. of the slopes obtained by the hyperbolic least-squares fit of each line in (a) to eqn. (9).

Table 2. Apparent inhibition constants for the products of *C. maenas* pyruvate kinase

The apparent inhibition constants were obtained by fitting the experimental data to the general rate equation for competitive, non-competitive, or parabolic competitive inhibition as appropriate (Cleland, 1967). The results are means $\pm$ S.E.M. determined by regression analysis.

Varied substrate	Fixed substrate	Product	Model fitted	Apparent constant	
A	B (35.8 μ M)	P	Non-competitive inhibition	K_i (intercept)	1.26 ± 0.23 mM
				K_i (slope)	2.75 ± 0.62 mM
B	A (104 μ M)	P	Competitive inhibition	K_i	2.18 ± 0.52 mM
A	B (2.27 mM)	Q	Non-competitive inhibition	K_i (intercept)	15.7 ± 3.9 mM
				K_i (slope)	7.89 ± 2.38 mM
B	A (104 μ M)	Q	Parabolic competitive inhibition	K_{i1}	1.18 ± 0.18 mM
				K_{i2}	8.61 ± 2.05 mM

replot from the data at the low phosphoenolpyruvate concentration are definitely a parabolic function of total ATP concentration, whereas those obtained at the high phosphoenolpyruvate concentration show less curvature and may be approximated by a straight line (Fig. 8). The inhibition of the enzyme by ATP when phosphoenolpyruvate is the variable substrate is shown in Fig. 9(a). There is no significant variation in the ordinate intercepts. The replot of the slopes, as a function of total ATP concentration, is probably curved, but over the concentration range studied it approximates to a straight line (Fig. 9b). Values of the apparent inhibition constants for the four inhibition patterns are given in Table 2.

The results indicated in Fig. 8 are those to be expected for the proposed mechanism if the concentration of ATP is significant compared with its inhibition constant K_{iq} . Reference to eqn. (6) shows that a parabolic relationship between the slopes of the primary plot (when ADP is the variable substrate) and ATP concentration is predicted. The slope replot has the following mathematical formulation:

$$\text{Slopes} = \frac{K_a}{V} \left(1 + \frac{B}{K_{ib}} + \frac{K_b A^2}{K_a K_{ia} B} + \frac{K_{ia} K_b}{K_a B} \right) + \frac{K_{ia} K_b}{V K_{iq} B} \cdot Q + \frac{K_{ia} K_b}{V K_{iq} K_{iq} B} \cdot Q^2$$

The apparently anomalous result of a linear slope replot obtained in the presence of a fixed high concentration of phosphoenolpyruvate (Fig. 8) can be readily explained. Note that in the equation above both the coefficient of the Q term (coeff. Q) and that of the Q^2 term (coeff. Q^2) are inversely dependent on the concentration of B . The dependence of the constant term, however, is a more complex function of B . Indeed between the two concentrations of B employed the value of this constant is virtually unchanged. The deviation of the data from linearity is

equal to (coeff. Q^2) $\cdot Q^2$. Thus over the same concentration range of Q (the conditions employed) reducing the value of coeff. Q^2 and of coeff. Q by increasing the concentration of B will result in the data approximating more nearly to a straight line. This is exactly the effect observed (Fig. 8).

Discussion

Recent evidence has indicated that the mammalian type-L pyruvate kinase is subject to a regulatory control mediated by a phosphorylation/dephosphorylation of the enzyme [for a review see Engstrom (1978) and references cited therein]. Nothing is known as to whether this type of mechanism occurs in *C. maenas*. However, the enzyme that was employed in the current study contained less than 5% of protein not associated with the enzyme-activity peak (determined by polyacrylamide-gel electrophoresis in the presence and absence of sodium dodecyl sulphate). The mammalian studies have not directly determined the ratio of phosphorylated/dephosphorylated enzyme in the purified enzyme. However, since up to 4 mol of phosphate can be incorporated into each mol of native tetramer, the implication is that the enzyme is isolated predominantly as the dephosphorylated form.

One other observation pertinent to the current investigation is that the addition of 5 μ M-fructose 1,6-bisphosphate overcomes the effect of phosphorylation, as monitored by the effects of phosphoenolpyruvate, ATP and alanine on the enzyme activity. For example, the phosphoenolpyruvate-saturation curve of the unphosphorylated rat liver enzyme is identical with that of the phosphorylated enzyme in the presence of 5 μ M-fructose 1,6-bisphosphate (Ekman *et al.*, 1976). Since the current series of experiments was conducted using a purified enzyme in the presence of 500 μ M-fructose 1,6-bisphosphate,

it has been assumed that only one form of the enzyme was present. The results obtained have been analysed on this assumption.

All the kinetic constants derived from the data presented in this paper are quoted in terms of total chemical concentration. This approach was decided on because of the uncertainty that exists as to the nature of the true substrates for pyruvate kinase. The major uncertainty is whether Mg^{2+} is a separate reactant in the reaction. Studies from various laboratories have led to conflicting conclusions on this point for both the type-L and type-M isoenzymes (Melchior, 1965; Mildvan & Cohn, 1966; Mildvan *et al.*, 1971; Ainsworth & Macfarlane, 1973; Macfarlane & Ainsworth, 1974). A steady-state kinetic analysis of the type-M pyruvate kinase from *C. maenas* indicated that two Mg^{2+} ions are required in the reaction mechanism (C. J. Newton, I. G. Giles & P. C. Poat, unpublished work). This is consistent with the dual bivalent-metal-ion binding of rabbit muscle pyruvate kinase detected by the use of Mn^{2+} and CrATP (Gupta *et al.*, 1976). The data on the *C. maenas* type-M isoenzyme can be taken to indicate that either MgADP or free ADP can bind to the enzyme to form a catalytically viable complex. In the latter case there can be a random addition of Mg^{2+} and ADP. It is a simple matter, however, to obtain the true kinetic constants once the true substrate species have been unambiguously identified, by multiplication by the appropriate constants. In spite of these limitations to the quantitative nature of the results, the qualitative aspects, which are of more importance in the present study, are valid.

The data presented are consistent with a rapid-equilibrium random mechanism with the formation of five dead-end complexes. Three of these dead-end complexes are the result of multiple bindings of the reactants of the enzyme. Substrate inhibition by ADP has been shown for the rabbit muscle enzyme (Janson & Cleland, 1974) and for the *C. maenas* muscle enzyme (Newton *et al.*, 1976). The latter enzyme also shows substrate inhibition by phosphoenolpyruvate. A study of the reverse reaction of rabbit muscle pyruvate kinase demonstrated directly that ATP was inhibitory at high concentrations (Giles *et al.*, 1976c). In the current study all three substrate inhibitions have been shown to occur in the

same enzyme. No evidence, however, was obtained to suggest that pyruvate has a similar effect. As a consequence these results may imply that substrate inhibition in this enzyme is dependent on the presence of a phosphate group.

The existence of two types of binding sites for ADP and phosphoenolpyruvate has been suggested as a result of studies of the protection of the rabbit muscle enzyme against inactivation by *p*-chloromercuribenzoate (Mildvan & Cohn, 1966). With a similar enzyme preparation, two types of binding sites for phosphoenolpyruvate were demonstrated by an analytical ultracentrifuge technique (Reynard *et al.*, 1961). More recently the X-ray-crystallographic studies of the cat muscle enzyme by Stammers & Muirhead (1975) have directly indicated the presence of two ADP-binding sites on the enzyme.

Qualitatively the product-inhibition patterns obtained are consistent with the proposed mechanism. The fact that phosphoenolpyruvate shows competitive inhibition against both products strongly suggests a random release of products. Table 2 summarizes the apparent inhibition constants found.

Two of the data sets are of no use in evaluating the true inhibition constants of the products for the reasons indicated in the Theory section. Calculation of the constants from the remaining data showed an apparent lack of internal consistency. This is illustrated in Table 3 where the data for the ATP inhibition against variable ADP have been fitted to eqn. (11). The values of V obtained, for example, in all but one case were much lower than the expected value of 1.21 obtained from the results of the initial-rate studies. The one case where there was close agreement was when a constant high concentration of phosphoenolpyruvate was present in the ATP-versus-ADP inhibition study. Reference to Table 3 also indicates that the values of K_{iq}^d and K_{iq} were 5–6-fold less when they were estimated from the data obtained at a high concentration of phosphoenolpyruvate than those found at a low concentration. Since it was unlikely that these discrepancies were the result of experimental error, an alternative explanation was sought. If any credence can be placed on the results, they suggest that there had been a change in affinity of the enzyme for ATP, although the concentration of fructose 1,6-bisphos-

Table 3. *ATP inhibition constants*

The constants were calculated from the data in Figs. 6 and 7 by fitting the experimental data to eqn. (11), utilizing the constants in Table 1. The constants are quoted in terms of the total chemical concentration.

Constant	[Phosphoenolpyruvate]	...	39.2 μM	2.27 mM
K_{iq}			0.353 mM	0.0613 mM
K_{iq}^d			12.6 mM	19.8 mM
K_{iq}^d			2.68 mM	0.443 mM
V			0.657	1.07

Fig. 10. Effect of phosphoenolpyruvate concentration on the initial rate of the forward reaction of *C. maenas* pyruvate kinase measured by using purified enzyme and enzyme present in a 100000 g supernatant of an hepatopancreas homogenate

The assay conditions were as described in Fig. 1 with a constant total ADP concentration of 50 μ M. The enzyme source was: O, 100000 g hepatopancreas supernatant; ●, purified enzyme.

phate was held at a saturating concentration of 500 μ M (Giles *et al.*, 1977).

Accordingly, the rate of the reaction was determined over a wide range of the substrate phosphoenolpyruvate in the presence of fructose 1,6-bisphosphate while the concentrations of Mg^{2+}_{free} and ADP_{total} were kept constant. The result is shown in Fig. 10. At the lowest concentrations of phosphoenolpyruvate the line is distinctly non-linear, even though 500 μ M-fructose 1,6-bisphosphate was present. When this experiment was repeated with a 100000 g supernatant of an hepatopancreas homogenate as the enzyme source there was no evidence of non-linearity (Fig. 10). Previous work has indicated that on purification this enzyme, like the mammalian type-L enzyme, loses its homotropic cooperative binding of fructose 1,6-bisphosphate (Giles *et al.*, 1977). This loss is perhaps the result of the loss of site-site communication. The current results suggest that in addition some of the binding sites for the positive modifier have also been masked, such that saturating the available sites does not cause complete activation. Addition of the substrate that also causes activation, phosphoenolpyruvate, however, does cause the conformational change to proceed to completion. As a consequence of this, the apparent affinity of the enzyme for its reactants must be dependent on the concentration of phosphoenolpyruvate.

These observations provide a ready explanation for the discrepancies seen in the kinetic constants shown in Table 3. When phosphoenolpyruvate was present at a high concentration the enzyme was fully activated and had a higher apparent affinity (as measured by K_{iq}) for ATP than at the low phosphoenolpyruvate concentration. The constant K_{Iq} does

Table 4. Best-estimate values of the product-inhibition constants for the fully activated enzyme

Constant	Value (mM)
K_{ip}	0.43
K_{ip}^d	0.808
K_{iq}	0.0613
K_{iq}^d	19.8
K_{Iq}^d	0.433
K'_{Ia}	0.145
K_{Ia}^d	0.559

not appear to be affected by the change in affinity of the enzyme. This suggests that when the second molecule of ATP binds to the enzyme it cannot bind purely to the phosphoenolpyruvate-binding site(s), since it would be reasonable to assume that the affinity for ATP at this site would also be changed. ATP therefore must decrease the phosphoenolpyruvate binding mainly by steric hinderance.

Since fructose 1,6-bisphosphate does not cause complete activation, the best estimates for the product-inhibition kinetic constants of the fully activated enzyme will be those obtained at a high concentration of phosphoenolpyruvate. These best estimates are indicated in Table 4, together with the values for pyruvate inhibition obtained from the apparent constants given in Table 2. These pyruvate constants were calculated by rearranging the hyperbolic form of eqn. (5) to the form of the equation for non-competitive inhibition and equating the apparent constants with the derived constants. This approach requires the least number of assumptions concerning values of constants (e.g. V is no longer involved). Finally two of the constants are derived from the equalities that exist between the various constants, namely:

$$K_{Ia} K_{Iq}^d = K_{Iq} K_{Ia}^d \text{ and } K_{Ia} K_{IP}^d = K_{IP} K'_{Ia}$$

A corollary of the above discussion is that the kinetic constants obtained in the absence of products most probably represent average values, as in the initial-rate studies the concentration of phosphoenolpyruvate had to be varied. Since the fructose 1,6-bisphosphate did not effect complete activation, one may expect to see non-linear replots with phosphoenolpyruvate as the variable substrate. The secondary replot of intercepts against reciprocal phosphoenolpyruvate concentration (Fig. 3a, derived from Fig. 1) shows deviations from linearity at the lowest three concentrations of the substrate. This is as expected if the enzyme was not completely activated at the low phosphoenolpyruvate concentrations. In order to estimate the kinetic constants of the fully activated enzyme, these data points were omitted from the subsequent numerical analysis.

As the existence on the enzyme of two sites for

ADP and two for ATP has been proposed, the nature of the ternary dead-end complex involving enzyme, ADP and ATP is ambiguous. It is possible that two such complexes occur, one of the form ATP-E-ADP and the other ADP-E-ATP. These cannot be distinguished by steady-state kinetics. Evidence is available, however, for the *C. maenas* leg muscle enzyme (Newton *et al.*, 1976) that suggests that the complex formed during ADP substrate inhibition is of the type MgADP-E-ADP. It is conceivable that the mixed-nucleotide dead-end complexes are MgATP-E-ADP and MgADP-E-ATP, where the Mg-chelated nucleotide is bound to its catalytically viable site. Under the conditions employed in the present study (8 mM-Mg²⁺_{free}) the magnesium chelates of ATP account for more than 99% of the total ATP added. Therefore as the magnesium chelate, MgATP²⁻, is the most abundant species of the product present the major dead-end complex is likely to be MgATP-E-ADP. Further, the dissociation of MgATP from this complex and from its binary complex with the enzyme are likely to be similar and dependent on the affinity state of the enzyme. Reference to Table 2 shows this to be the case.

One final observation that is consistent with the proposed mechanism is the reversal of inhibition in the presence of pyruvate when phosphoenolpyruvate is held constant at a saturating concentration (2.32 mM) and ADP is the variable substrate (Giles *et al.*, 1975). Under these conditions the apparent K_m for ADP is 20 times larger than is expected. This can now be explained in terms of the substrate inhibition caused by the high concentration of phosphoenolpyruvate.

In conclusion it is noteworthy that the activated form of this type-L pyruvate kinase has many common facets with the mechanism of the type-M enzyme from rabbit muscle. These common areas include the apparent validity of the rapid-equilibrium assumption in the forward reaction, substrate inhibition, random binding of substrates and products, and dead-end-complex formation.

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