Kinetics and Mechanism of Action of Muscle Pyruvate Kinase

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1. The mechanism of rabbit muscle pyruvate kinase was investigated by measurements of fluxes, isotope trapping, steady-state velocity and binding of the substrates. All measurements were made at pH 8.5 in Tris/HCl buffer and at 5 mM-free Mg^{2+} . 2. Methods of preparing $[^{32}P]$ phosphoenolpyruvate from $[^{32}P]P_i$ in high yield and determining $[^{32}P]$ -phosphoenolpyruvate and $[8-^{14}C]ADP$ are described. 3. The ratio Flux of ATP to ADP/Flux of ATP to phosphoenolpyruvate (measured at equilibrium) increased hyperbolically with ADP concentration from unity to about 2.1 at 2 mM-ADP, but was unaffected by phosphoenolpyruvate concentration. Since the ratio is greater than unity, one pathway for the addition of substrates must involve phosphoenolpyruvate adding first to the enzyme in a rate-limiting step. However, the substrates must also add in the alternative order, because of the non-linear increase in the ratio with ADP concentration and because the rate of increase is very much less than that predicted from the steady-state velocity data for an ordered addition. The lack of influence of phosphoenolpyruvate on the ratio is consistent with the rapid addition of ADP in the alternative pathway. At low ADP concentrations the alternative pathway contributes less than 33% to the total reaction. 4. Isotope trapping was observed with $[^{32}P]$ phosphoenolpyruvate, confirming that when phosphoenolpyruvate adds first to the enzyme it is in a rate-limiting step. The release of phosphoenolpyruvate from the ternary complex must also be a slow step. Trapping was not observed with $[8-^{14}C]ADP$, hence the addition of ADP to the free enzyme must be rapid unless its dissociation constant is very large (>20 mM). 5. Binding studies showed that 4 mol of $[^{32}P]$ phosphoenolpyruvate binds to 1 mol of the enzyme, probably unligated to Mg^{2+} , with a dissociation constant appropriate to the mechanism indicated above. Binding of $[8-^{14}C]ADP$ could not be detected, and hence the binding of ADP occurs by a low-affinity step. The latter is also demanded by the steady-state velocity data. 6. The ratio Flux of phosphoenolpyruvate to ATP/Flux of phosphoenolpyruvate to pyruvate (determined from the incorporation of label into phosphoenolpyruvate from $[3-^{14}C]$ -pyruvate or $[\gamma-^{32}P]ATP$ during the forward reaction) did not differ significantly from unity. Steady-state velocity data predicted grossly different flux ratios for ordered dissociations of the products, and the results indicate that the dissociation must be rapid and random. The data also exclude a Ping-Pong mechanism. 7. Permissible rate constants for the above mechanism are calculated. The results indicate a high degree of cooperativity in binding, whatever the order of addition of substrate.

Pyruvate kinase (EC 2.7.1.40) catalyses the formation of pyruvate and ATP from phosphoenolpyruvate and ADP and is an important enzyme in the regulation of glycolysis and gluconeogenesis (Llorente *et al.*, 1970; Liao & Atkinson, 1971). The enzymes, which have been isolated from mammalian tissues (Tietz & Ochoa, 1958; Irving & Williams, 1973; Nicholas & Bachelard, 1974), require Mg^{2+} or Mn^{2+} and K^+ for activity. They show hyperbolic or sigmoidal steady-state velocity kinetics with respect

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to the substrate phosphoenolpyruvate and have been classified as M type, having hyperbolic kinetics, and L type, having sigmoidal kinetics and requiring fructose 1,6-bisphosphate for activation.

The mechanisms of the mammalian and yeast enzymes have been investigated, but results do not support a single mechanism. Initial-velocity studies have been consistent with a rapid-equilibrium random mechanism for the muscle M-type enzyme (Reynard *et al.*, 1961; Ainsworth & MacFarlane, 1973), an ordered addition of substrates and dissociation of products for the yeast enzyme (MacFarlane & Ainsworth, 1972) and a Ping-Pong

mechanism involving a phosphoenzyme for the liver L-type enzyme (MacFarlane & Ainsworth, 1974). There are difficulties, however, in the interpretation of initial-velocity studies, and we are therefore studying the mechanism of these enzymes with a variety of techniques, including flux measurements with labelled substrates (Britton, 1966; Britton & Dann, 1978), isotope-trapping (Rose *et al.*, 1974), binding and initial-velocity studies. The present paper describes the results obtained on the rabbit muscle enzyme.

Materials and Methods

Materials

Substrates and enzymes were obtained from Boehringer Corp. (London) Ltd. (London W.5, U.K.); ADP, ATP, NADH, NADP⁺ and pyruvate were sodium salts; phosphoenolpyruvate and glyceraldehyde 3-phosphate diethyl acetal were cyclohexylammonium salts. Scintillation chemicals were from Packard Instruments (Wembley, Middx., U.K.) and Rohm and Haas (U.K.) Ltd. (Croydon, Surrey, U.K.). All other chemicals were reagent grade or better and were obtained from BDH Chemicals (Poole, Dorset, U.K.). For the kinetic experiments enzyme suspensions were centrifuged and the sedimented enzymes dissolved to the original volume with water.

[8-¹⁴C]ADP (ammonium salt), phospho[3-¹⁴C]-enolpyruvate (cyclohexylammonium salt) and [³²P]-orthophosphate (in dilute HCl, pH 2–3) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Phospho[3-¹⁴C]enolpyruvate was dissolved in water, and the solution divided into small portions and freeze-dried. All labelled compounds were stored at –20°C. [³²P]Phosphoenolpyruvate was prepared from [³²P]P_i and glyceraldehyde 3-phosphate by an extension of the method of Schendel & Wells (1973). A 0.1 ml portion (1 mCi) of [³²P]P_i, pH 2–3, in a conical centrifuge tube, was dried in a vacuum desiccator over NaOH. In a second tube, 95 µl of a solution containing (final concentrations) 26 mM-Tris/HCl buffer, pH 9.0, 1.05 mM-MgCl₂, 0.26 mM-KH₂PO₄, 0.53 mM-ADP, 1.27 mM-NAD⁺, 0.25 mg of Thiazolyl Blue/ml, 0.63 mg of promethazine/ml and 10.5 mM-sodium pyruvate was prepared and transferred to the tube containing the [³²P]P_i with a Pasteur pipette. A 2 µl portion of pyruvate kinase [10 mg/ml in 3.2 M-(NH₄)₂SO₄] and 2 µl of a mixed enzyme preparation [glyceraldehyde 3-phosphate dehydrogenase (1 mg/ml) and 3-phosphoglycerate kinase (5 mg/ml) in 3.2 M-(NH₄)₂SO₄] were added. A 20 µl portion of glyceraldehyde 3-phosphate (16.3 mM), prepared immediately before use by dissolving 3.26 µmol of the diacetal in 100 µl of 0.2 M-HCl,

heating the solution at 80°C for 2 min, and, after cooling, neutralization with 100 µl of 0.2 M-KHCO₃, was added and the solution was incubated in the dark at room temperature (24°C) for 3–4 h. A 10 µl portion of EDTA (50 mM) was added to stop the reaction. To isolate [³²P]phosphoenolpyruvate, 10 µl of the incubation mixture was applied as a 5 cm strip on a thin-layer cellulose plate and chromatographed with di-isopropyl ether/formic acid (3:2, v/v) (Eggleston & Hems, 1952). The labelled phosphoenolpyruvate was identified with a Geiger-Müller tube and the strip cut out of the plate. The phosphoenolpyruvate was chromatographed to the margin of the strip with water and then extracted with 1 ml of 0.2 mM-phosphoenolpyruvate. The reaction proceeded to completion with little [³²P]P_i remaining (<10%), but it was important that the chromatography and extraction be carried out without delay to avoid hydrolysis. The purity of preparations was measured by reaction with pyruvate kinase and excess of ADP and counting the radioactivity of samples of the reaction mixture for phosphoenolpyruvate as P_i (see below). Samples were also chromatographed, as described above. With both techniques not less than 95% of the radioactivity behaved as phosphoenolpyruvate.

Methods

[³²P]Phosphoenolpyruvate was measured by precipitation with ammonium molybdate as follows: a sample (50 µl) containing the label was added to a mixture of 10 µl of saturated HgCl₂ and 10 µl of 20 mM-K₂HPO₄. Hydrolysis of phosphoenolpyruvate to P_i was almost immediate under these conditions. After about 5 min, 1.25 ml of a solution containing 0.16 M-HClO₄, 2 mM-ammonium molybdate and 4 mM-triethylamine/HCl buffer, pH 6, was added (Sugino & Miyoshi, 1964) and the solution was stored for 1 h at 0–4°C. After centrifugation, the supernatant was removed and a further 1.25 ml of the phosphate-precipitating mixture added; the supernatant was again removed, and the precipitate was dissolved in 300 µl of NaOH and transferred with two washes of 0.5 ml of water to vials. A 10 ml portion of scintillation mixture [666 ml of toluene, 333 ml of Triton X-100, 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene] was added and the radioactivity in the vials was counted in a Packard model 3000 Tri-Carb liquid-scintillation counter (Packard Instruments). Recovery of ³²P-labelled substrate was not less than 95%.

¹⁴C-labelled compounds were separated on PEI-cellulose (polyethyleneimine-cellulose) thin-layer plates (Andeman and Co., East Moseley, Surrey, U.K.) and their radioactivities counted as described previously (Dann & Britton, 1977). The recovery of ¹⁴C-labelled substrates was not less than 95%.

Pyruvate kinase activities were measured spectrophotometrically (Tietz & Ochoa, 1958) in a reaction solution of 3 ml in a cuvette of 10 mm path-length containing the following (final concentrations): 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 5 mM-MgCl₂, 0.16 mM-NADH, 1.2 mM-ADP, 0.6 mM-phosphoenolpyruvate and 50 μ g of lactate dehydrogenase/3 ml. The specific activity of different batches was in the range 50–160 units (μ mol of substrate transformed)/mg, and the enzyme ran as a single band on zone electrophoresis and on polyacrylamide gels (Dann & Britton, 1974).

Rapid equilibrium dialysis was performed in a Technilab dialysis cell (Bolab Inc., Derry, NH, U.S.A.) as described by Colowick & Womack (1969). The flow rate through the lower half of the cell was maintained at 6 ml/min and 2 ml fractions were collected. Corrections for transfer of labelled substrate across the membrane (about 1.7% of the total radioactivity) and for dilution of the label by successive additions of unlabelled substrates were applied. The membrane was cut from dialysis tubing, treated as described by Garland *et al.* (1964) and stored in water at 4°C.

Isotope trapping was conducted as described by Rose *et al.* (1974). A 0.1 ml portion of a 'pulse' solution (1.09), containing pyruvate kinase, 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 5 mM-MgCl₂ and either 20 μ M-[³²P]phosphoenolpyruvate or 87 μ M-[8-¹⁴C]ADP, was rapidly added to 1 ml of a 'chase' solution of unlabelled ADP and phosphoenolpyruvate, which was vigorously stirred at room temperature (23–26°C). The 'chase' solution for [³²P]phosphoenolpyruvate trapping contained 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 62 mM-MgCl₂ (5 mM free Mg²⁺), 10 mM-ADP and 100 mM-phosphoenolpyruvate at an ionic strength of 0.42 mol/l. For [8-¹⁴C]ADP trapping it contained 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 108 mM-MgCl₂ (5 mM free Mg²⁺), 100 mM-ADP and 10 mM-phosphoenolpyruvate. Both solutions were finally adjusted to pH 8.5 with NaOH. After approx. 1 s 1 ml of 1 M-HClO₄ was added, and after 5 min 1 ml of the mixture was removed and neutralized with 0.5 ml of 1 M-KHCO₃. The solution was centrifuged to remove KClO₄; ATP, [³²P]phosphoenolpyruvate, [8-¹⁴C]-ADP and [8-¹⁴C]ATP were then determined as described below. [γ -³²P]ATP was determined by counting the radioactivity of 1 ml of the supernatant obtained from the phosphate precipitation of [³²P]-phosphoenolpyruvate; the γ -phosphate group of ATP is not hydrolysed under these conditions (Sugino & Miyoshi, 1964).

Steady-state velocity and flux measurements

These were carried out at 25°C in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl. Sufficient

MgCl₂ was added to give a free Mg²⁺ concentration of 5 mM and tetraethylammonium chloride was added to give an ionic strength of 0.2 mol/l. Bound Mg²⁺ was calculated from the dissociation constants for Mg²⁺-phosphoenolpyruvate (Wold & Ballou, 1957), Mg²⁺-ATP and Mg²⁺-ADP (Phillips *et al.*, 1966). The Mg²⁺-nucleotide solutions were adjusted to pH 8.5 with NaOH.

Steady-state velocities were measured by the addition of NADH and lactate dehydrogenase or of glucose (1.9 mM), hexokinase and glucose 6-phosphate dehydrogenase and recording the change in *A*₃₄₀ (SP.1800 spectrophotometer; Pye-Unicam, Cambridge, U.K.). In both cases sufficient coupling enzymes were added for the rate to be independent of their quantity both at high and at low substrate concentration. Fresh solutions of ATP contained 0.8% ADP by assay with pyruvate kinase. All ATP solutions were therefore assayed for ADP and allowances made for the ADP content where necessary. Straight lines were fitted to experimental points in Lineweaver-Burk plots by using a weighted linear regression program with a weighting factor of (velocity)⁻⁴ (Wilkinson, 1961). Intersection of the lines at a common point was not a condition of the program.

The ratio Flux of ATP to phosphoenolpyruvate/Flux of ATP to ADP, and its reciprocal, were measured at constant pyruvate concentration (40 mM) and at different concentrations of ADP or phosphoenolpyruvate, the concentration of the second substrate being kept constant at 0.1 mM. Pyruvate kinase was incubated with an equilibrium mixture of substrates for sufficient time to ensure complete equilibrium (30 min). [³²P]Phosphoenolpyruvate and [8-¹⁴C]ADP, in a small volume, were added and 50 μ l samples were taken at intervals up to 20 min. Then 0.2 mg of pyruvate kinase was added and two more samples were taken after 5 and 10 min: these samples allowed measurement of the equilibrium values of [³²P]phosphoenolpyruvate and [8-¹⁴C]-ADP. The incorporation of ¹⁴C and ³²P into ATP was exponential, and the half-times ranged between 3 and 20 min, depending on the concentrations of substrates and enzyme. Owing to the position of the equilibrium, the final radioactivities in the ADP and phosphoenolpyruvate were only a small fraction of those added initially. Each 50 μ l sample was added to 20 μ l of a mixture of saturated HgCl₂ and 20 mM-K₂HPO₄ (1:1, v/v) as described above. After 5 min, 5 μ l was withdrawn and spotted as a 1 cm strip on PEI-cellulose for the separation of ¹⁴C-labelled substrates; to the remainder was added the phosphate-precipitating mixture as described above. The equilibrium concentrations of substrates were determined as follows. For phosphoenolpyruvate and pyruvate, 100 μ l and 5 μ l samples respectively were added to 50 μ l and 10 μ l of 1 M-HCl to inactivate the

enzyme. After 10 min the solution was neutralized with 1 M-KHCO₃, and 2.5 ml of 50 mM-triethanolamine buffer, pH 7.6 (containing 50 mM-KCl and 5 mM-MgCl₂) was added. Similarly, for ADP 100 μ l was added to 100 μ l of 9 M-urea, and for ATP 10 μ l was added to 20 μ l of ethanol. After 15 min, 2.5 ml of buffer was added as before. Substrate concentrations were determined spectrophotometrically by coupling to lactate dehydrogenase (for phosphoenolpyruvate, pyruvate and ADP) or to hexokinase and glucose 6-phosphate dehydrogenase (for ATP). In the measurement of phosphoenolpyruvate and ADP, pyruvate was first removed in the spectrophotometer cell by reaction with NADH. Conversion of pyruvate into lactate was shown to remain quantitative even with the large amounts of lactate that accumulated. Fluxes were calculated as described by Britton & Clarke (1968) by using a linear regression to calculate the gradient of the logarithmic plots.

The ratio Flux of phosphoenolpyruvate to pyruvate/Flux of phosphoenolpyruvate to ATP was determined from the rate of incorporation of label into phosphoenolpyruvate from [3-¹⁴C]pyruvate while the reaction proceeded in a forward direction (Britton & Dann, 1978). The ATP formed was immediately converted into ADP with excess of hexokinase. [3-¹⁴C]Pyruvate (70 μ M) was generated from 2 μ Ci of phospho[3-¹⁴C]enolpyruvate (specific radioactivity 9.0 mCi/mmol) in the presence of 133 μ M-ADP, 10 mM-glucose, 50 μ M-pyruvate, 20 μ g of hexokinase and 4.5 μ g of pyruvate kinase in a volume of 1.7 ml. After incubation at 25°C for 10 min, 300 μ l of 100 mM-phosphoenolpyruvate was added, and 20 μ l samples were withdrawn at intervals and added to 20 μ l of 1 M-HCl to stop the reaction. After neutralization with 1 M-KHCO₃, 50 μ l was taken for determination of glucose 6-phosphate and 5 μ l was spotted on PEI-cellulose for isolation of phospho-[3-¹⁴C]enolpyruvate as described above. Generation of [3-¹⁴C]pyruvate *in situ* in this way avoided difficulties due to the instability of pyruvate.

Similarly, the ratio Flux of phosphoenolpyruvate to ATP/Flux of phosphoenolpyruvate to pyruvate was determined with [γ -³²P]ATP. The pyruvate produced in the reaction was converted into lactate with lactate dehydrogenase. [γ -³²P]ATP (2 μ M) was generated from [³²P]phosphoenolpyruvate in the presence of 20 μ M-ADP, 5 mM-NADH, 20 μ g of lactate dehydrogenase and 50 μ g of pyruvate kinase in a volume of 2.7 ml. After 10 min incubation at 25°C, 320 μ l of a mixture of phosphoenolpyruvate, ATP and glucose was added to give final concentrations of 10 mM, 560 μ M and 10 mM respectively. From this solution 500 μ l was withdrawn as a control; to the remainder, 0.67 μ g of hexokinase was added to start the reaction. Then 100 μ l samples were withdrawn at intervals and each was added to 50 μ l of 1 M-HCl to inactivate the enzyme. After 5 min each

solution was neutralized with 50 μ l of 1 M-KHCO₃, and 50 μ l samples were taken for counting of the radioactivity of [³²P]phosphoenolpyruvate and for the spectrophotometric determination of NADH and ADP. Under the conditions of the experiment, hexokinase was rate-limiting, and the concentration of ADP did not exceed 30 μ M.

Protein concentrations were measured by the method of Lowry *et al.* (1951), with human plasma albumin as standard. A molecular weight of 237 000 was taken for rabbit muscle pyruvate kinase (Cottam *et al.*, 1969).

Results

All flux and steady-state velocity measurements were carried out at 25°C in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl and sufficient MgCl₂ to give a free Mg²⁺ concentration of 5 mM. Tetraethylammonium chloride was added to maintain the ionic strength at 0.2 mol/l. Under these conditions and by using total concentrations of substrates and products the apparent equilibrium constant was 2125 ± 81 (mean $\pm$ S.E.M., 12 observations).

Measurements of the ratio Flux of ATP to ADP/Flux of ATP to phosphoenolpyruvate

Measurements were made at different ADP concentrations while the pyruvate and phosphoenolpyruvate concentrations were kept constant (Fig. 1a). The ATP concentration was adjusted in each case to maintain equilibrium. The ratio increased with ADP from unity to a value of about 2.1 at an ADP concentration of 2 mM. Although the scatter of points is considerable, the increase was not linear, with a tendency for the ratio to plateau at higher ADP values. Additional points are shown in Fig. 1(a) measured at other phosphoenolpyruvate concentrations (0.29–0.34 mM), since it is shown below that the ratio is independent of the concentration of this substrate. The points fit well with those measured at constant phosphoenolpyruvate concentration and provide further evidence that the increase in the ratio is not a linear function of the ADP concentration.

The above ratio was also measured at different phosphoenolpyruvate concentrations while pyruvate and ADP concentrations were kept constant, the ATP concentration being adjusted to maintain equilibrium. The reciprocal of the ratio was independent of phosphoenolpyruvate concentration (Fig. 1b), with an average value near to unity. If the flux ratio is independent of phosphoenolpyruvate concentration, it should depend only on ADP concentration, since the pyruvate concentration was kept constant [see eqn. (3) in Britton & Dann (1978), where A is phosphoenolpyruvate, B is ADP, P is ATP

Fig. 1. Flux ratios related to the addition of substrates

(a) The ratio Flux of ATP to ADP/Flux of ATP to phosphoenolpyruvate plotted as a function of the concentration of ADP. Pyruvate concentration was maintained constant at 40 mM and the ATP concentration was adjusted to maintain equilibrium. □, Experiments at 0.1 mM-phosphoenolpyruvate concentration; △, experiments at 0.29–0.34 mM-phosphoenolpyruvate concentration. Line II (---) is the theoretical line for an ordered mechanism with phosphoenolpyruvate adding first to the enzyme, calculated from the Dalziel coefficients. Line I (—) is a theoretical line for a random addition of substrates with the addition of phosphoenolpyruvate as a rate-limiting step (Scheme 3 of Fig. 1 in Britton & Dann, 1978). A flux ratio of 2.1 at an ADP concentration of 2 mM and a value of $K_B = 2$ mM have been assumed. (b) The ratio Flux of ATP to phosphoenolpyruvate/Flux of ATP to ADP plotted as a function of the concentration of phosphoenolpyruvate. Pyruvate concentration was maintained constant at 40 mM and the ATP concentration was adjusted to maintain equilibrium. □, Experiments at 0.1 mM-ADP concentration; △, experiments at 0.15–0.25 mM-ADP concentration. In the latter case the experimental flux ratios were multiplied by a factor derived from line I (a) to obtain a value corresponding to an ADP concentration of 0.1 mM. Line III represents the theoretical value of the flux ratio at an ADP concentration of 0.1 mM from line I (a) assuming that the ratio is independent of the phosphoenolpyruvate concentration. All experiments were carried out at 25°C in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl and sufficient $MgCl_2$ to give a concentration of free Mg^{2+} of 5 mM. Tetraethylammonium chloride was added to give 10.2. Human plasma albumin was added to a concentration of 0.6 mg/ml.

and Q is pyruvate]. Line III (Fig. 1b) represents the ratio calculated for the ADP concentration (0.1 mM) by using the theoretical line I (Fig. 1a). Fig. 1(b) also contains results collected at different ADP concentrations (0.15–0.25 mM). The measured flux ratios with these data were adjusted to give predicted values at an ADP concentration of 0.1 mM by means of line I (Fig. 1a).

Measurements of the ratio Flux of phosphoenolpyruvate to pyruvate/Flux of phosphoenolpyruvate to ATP

This ratio was determined from the incorporation of label into phosphoenolpyruvate from pyruvate with the reaction proceeding in the forward direction. Since the addition of phosphoenolpyruvate and ADP to the enzyme might be ordered [see Britton & Dann, (1978) and Appendix II], it was important, if incorporation into phosphoenolpyruvate was to be

detected, that the concentration of ADP should be low. It was therefore restricted to 113 μ M and was continuously regenerated by excess of hexokinase and glucose. In three separate experiments, one illustrated (Fig. 2a), no incorporation of ^{14}C into phosphoenolpyruvate was observed. The ratio Flux of phosphoenolpyruvate to pyruvate/Flux of phosphoenolpyruvate to ATP was therefore not significantly greater than unity under the experimental conditions. To set limits on this ratio it may be assumed that the ratio increases linearly from unity with pyruvate concentration as for an ordered dissociation of products. If the flux ratio equals $(1 + 0.008[Pyr])$, where $[Pyr]$ is the concentration of pyruvate (mM), then it may be calculated (see Appendix II) that at least 3% incorporation into phosphoenolpyruvate would have been observed when the phosphoenolpyruvate concentration had fallen to 11 mM and 4% incorporation when the concentration had reached 9 mM. Any incorporation was substantially less than

Fig. 2. Flux ratios related to the dissociation of products

(a) The ratio Flux of phosphoenolpyruvate to pyruvate/Flux of phosphoenolpyruvate to ATP determined by the incorporation of label from $[3\text{-}^{14}\text{C}]\text{pyruvate}$ into phosphoenolpyruvate. The reaction was carried out in the forward direction, and ATP formed was reconverted into ADP with glucose and an excess of hexokinase. The incorporation of label ($\circ$) is expressed as a percentage of the label originally present in the $[3\text{-}^{14}\text{C}]\text{pyruvate}$ and is plotted against the phosphoenolpyruvate concentration. The concentration of ADP was $113\text{ }\mu\text{M}$. The $[3\text{-}^{14}\text{C}]\text{pyruvate}$ ($70\text{ }\mu\text{M}$) was generated *in situ* from phospho $[3\text{-}^{14}\text{C}]\text{enolpyruvate}$ as described in the Materials and Methods section. The continuous line I represents the expected incorporation for an ordered dissociation of products with pyruvate first to dissociate from the ternary complex, assuming that there is an ordered addition of substrates with the phosphoenolpyruvate first to add to the enzyme. The continuous line II represents the expected incorporation for an ordered dissociation if there is a rapid random addition of substrates or an ordered addition with ADP first to add to the enzyme. The incorporation for an ordered dissociation when there is a random addition of substrates that is not rapid may be expected to lie within the stippled area. The incorporation for a Ping-Pong mechanism would be 44% at 14 mM -phosphoenolpyruvate and 85% at 9 mM -phosphoenolpyruvate. Zero incorporation of label into phosphoenolpyruvate indicates a flux ratio of unity. (b) The ratio Flux of phosphoenolpyruvate to ATP/Flux of phosphoenolpyruvate to pyruvate determined by the incorporation of label from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into phosphoenolpyruvate. The incorporation of label into phosphoenolpyruvate ($\bullet$) is expressed as a percentage of the label originally present in the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and is plotted against the phosphoenolpyruvate concentration. The reaction was carried out in the forward direction with the addition of lactate dehydrogenase to convert pyruvate into lactate. ADP was generated from ATP by adding glucose and hexokinase. The quantity of the latter enzyme was rate-limiting, so that the concentration of ADP did not exceed $30\text{ }\mu\text{M}$. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was produced *in situ* from $[^{32}\text{P}]\text{phosphoenolpyruvate}$ as described in the Materials and Methods section, and unlabelled ATP was added to give a concentration of 0.56 mM . The continuous line III represents the expected incorporation of label into phosphoenolpyruvate for an ordered dissociation with ATP first to leave the ternary complex, assuming an ordered addition of substrates with phosphoenolpyruvate first to add to the enzyme. The continuous line IV is the expected incorporation for an ordered dissociation, if the addition of substrates is rapid and random or if there is an ordered addition with ADP first to add to the enzyme. The incorporation for an ordered dissociation when the addition of substrates is random but not rapid may be expected to lie in the hatched area between the lines. Zero incorporation of label into phosphoenolpyruvate indicates a flux ratio of unity. The theoretical lines were calculated from the steady-state velocity data (see under 'Interpretation of results' in the Discussion section). The reaction conditions were as described in Fig. 1.

this and the flux ratio therefore must have been proportionately smaller. Since the maximum pyruvate concentration was 6.2 mM , it follows that the maximum flux ratio must have been less than 1.050. If the dissociation of the products should be random, the flux ratio will not be a linear function of the pyruvate concentration, as assumed above, but will rise hyperbolically to a plateau. Under these conditions the incorporation of ^{14}C into phosphoenolpyruvate would have been greater for the same maximum flux ratio (see Appendix II). Therefore 1.050 represents a maximum limit for the flux ratio.

The ratio Flux of phosphoenolpyruvate to ATP/Flux of phosphoenolpyruvate to pyruvate was determined from the incorporation of label from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into phosphoenolpyruvate. Lactate dehydrogenase and NADH were added to remove pyruvate, and the ATP concentration was maintained at $560\text{ }\mu\text{M}$. To facilitate the incorporation of label into phosphoenolpyruvate, the ADP concentration was maintained at less than $30\text{ }\mu\text{M}$ by adding glucose and a rate-limiting quantity of hexokinase. No incorporation of label into phosphoenolpyruvate was found (Fig. 2b), and this result was confirmed in

Fig. 3. Michaelis-Menten kinetics of pyruvate kinase

(a) Reciprocal plots of velocity against the concentration of phosphoenolpyruvate. The concentrations of ADP were as follows: $\bullet$, $33 \mu\text{M}$; $\blacktriangle$, $66 \mu\text{M}$; $\blacktriangledown$, $100 \mu\text{M}$; $\circ$, $300 \mu\text{M}$. (b) Reciprocal plots of velocity against the concentration of ADP. The concentrations of phosphoenolpyruvate were as follows: $\bullet$, $33 \mu\text{M}$; $\blacktriangle$, $66 \mu\text{M}$; $\blacktriangledown$, $100 \mu\text{M}$; $\circ$, $300 \mu\text{M}$. The reaction was carried out at 25°C and at I 0.2 in a volume of 3 ml containing the following: 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 5 mM free Mg^{2+} , $66 \mu\text{M}$ -NADH, 25 μg of lactate dehydrogenase and 0.6 mg of human plasma albumin. The reaction was started by the addition of 0.1 μg of pyruvate kinase. Straight lines were fitted by using a weighted linear-regression program, without regard to their point of intersection.

two similar experiments. From the arguments used above, a ratio Flux of phosphoenolpyruvate to ATP/Flux of phosphoenolpyruvate to pyruvate of 1.003 at $560 \mu\text{M}$ -ATP would have led to at least 3% incorporation of label into phosphoenolpyruvate. This value therefore represents the maximum value of the ratio. This conclusion is independent of any assumptions about the dissociation of the products, since the ATP concentration was maintained constant throughout.

Steady-state velocity kinetics

Measurements of the variation in the initial velocity with phosphoenolpyruvate and ADP gave linear reciprocal plots (Fig. 3). Values of ϕ_0 , ϕ_A , ϕ_B and ϕ_{AB} (Table 1) were obtained from replots of slopes and intercepts.

Reciprocal plots of the initial velocity against phosphoenolpyruvate concentration in the presence of a product were linear (Figs. 4a and 4b). An ordered dissociation of products was assumed, and two values of ϕ_{AB}^* (Table 1) were calculated from the slope replots (Fig. 4 inset) for pyruvate or ATP dissociating first from the ternary complex [see eqn. (18) in Britton & Dann (1978)]. The dissociation constants for a rapid and random dissociation [eqn. (1) of Appendix I] were also calculated (Fig. 6).

Substrate binding

Rapid-equilibrium-dialysis experiments with $[^{32}\text{P}]$ -phosphoenolpyruvate were conducted at room temperature (23 – 26°C) in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl and 5 mM-free Mg^{2+}

Table 1. ϕ values and slope replots m_P and m_Q for rabbit muscle pyruvate kinase at 25°C , pH 8.5, Mg^{2+} (free) 5 mM and I 0.2

Values, obtained by least-squares fitting of the data in Figs. 3 and 4, are expressed per μg of enzyme. A is phosphoenolpyruvate, B is ADP, P is ATP and Q is pyruvate, except where indicated. The values in parentheses refer to those obtained from a duplicate set of experiments (not illustrated).

$$\begin{aligned}\phi_0 &= 5.38 \text{ (5.50) } \mu\text{g}/\mu\text{mol} \\ \phi_A &= 0.175 \text{ (0.177) } \mu\text{g} \cdot \text{mm}/\mu\text{mol} \\ \phi_B &= 0.939 \text{ (0.949) } \mu\text{g} \cdot \text{mm}/\mu\text{mol} \\ \phi_{AB} &= 0.0767 \text{ (0.0724) } \mu\text{g} \cdot \text{mm}^2/\mu\text{mol} \\ m_Q &= 0.177 \text{ (0.133) } \mu\text{g}/\mu\text{mol} \text{ (B) } = 0.1 \text{ mM; Fig. 4} \\ m_P &= 0.690 \text{ (0.705) } \mu\text{g}/\mu\text{mol} \text{ (B) } = 0.05 \text{ mM; Fig. 4} \\ \phi_{AB}^* \text{ (pyruvate)} &= 0.0117 \text{ (0.0133) } \mu\text{g} \cdot \text{mm}/\mu\text{mol} \\ \phi_{AB}^* \text{ (ATP)} &= 0.0345 \text{ (0.0353) } \mu\text{g} \cdot \text{mm}/\mu\text{mol}\end{aligned}$$

* Assuming an ordered dissociation with pyruvate dissociating first (P is pyruvate).

† Assuming an ordered dissociation with ATP dissociating first (P is ATP).

and at I 0.2. A typical experiment is shown in Fig. 5(a). The Scatchard plots gave a binding constant of $65 \pm 3.1 \mu\text{M}$ (mean $\pm$ S.E.M., three observations) and four binding sites per molecule of enzyme. In the absence of Mg^{2+} (not shown), the binding constant was decreased, and values of $35 \mu\text{M}$ and $49 \mu\text{M}$ were obtained in duplicate observations. In all experiments the enzyme was fully active at the end of the dialysis, and an assay for $[^{32}\text{P}]\text{P}_i$ with the phosphate-precipitating mixture showed no measurable hydrolysis ($<1\%$) of phosphoenolpyruvate.

Fig. 4. Inhibition of pyruvate kinase by pyruvate or ATP

(a) Pyruvate concentrations were as follows: ○, 0 mM; △, 5 mM; ●, 10 mM; □, 15 mM; ■, 20 mM. The reaction was carried out at 25°C and at *I* 0.2 in a volume of 3 ml containing the following: 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 5 mM free Mg^{2+} , 0.1 mM-ADP, 1.9 mM-glucose, 85 μ M-NADP⁺, 20 μ g of hexokinase, 10 μ g of glucose 6-phosphate dehydrogenase and 0.6 mg of human plasma albumin. The reaction was started by the addition of 0.25 μ g of pyruvate kinase. (b) ATP concentrations were as follows: ●, 0 mM; △, 0.55 mM; □, 1.30 mM; ○, 2.60 mM; ■, 3.7 mM; ▲, 5.55 mM. The reaction was carried out at 25°C and at *I* 0.2 in a volume of 3 ml containing the following: 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 5 mM free Mg^{2+} , 50 μ M-ADP, 66 μ M-NADH, 25 μ g of lactate dehydrogenase and 0.6 mg of human plasma albumin. The reaction was started by the addition of 0.25 μ g of pyruvate kinase. Straight lines were fitted by using a weighted linear-regression program, without regard to their point of intersection. Replots of the slopes of the lines are shown inset.

In contrast, no binding of [8-¹⁴C]ADP could be detected in duplicate experiments either in the presence of 5 mM-free Mg^{2+} (Fig. 5b) or in the absence of Mg^{2+} . A minimum value for the dissociation constant of ADP from the enzyme can be established from these results as follows.

Let

$$\frac{[E-ADP]}{[ADP_T]} = r \quad (1)$$

where [E-ADP] represents the concentration of the enzyme-ADP complex and [ADP_T] the total concentration of ADP, both free and bound. Since no binding was detected $r \ll 1$, under these conditions:

$$K_{ADP} = \{(1-r)[E_T]/r\} - [ADP_T] \quad (2)$$

where K_{ADP} is the dissociation constant for ADP and [E_T] the total concentration of enzyme. r is at a maxi-

mum before the addition of unlabelled ADP, and a 5% change in radioactivity would have been detected. Thus, if $r < 0.05$, [ADP_T] = 8.6 μ M and [E_T] = 17 μ M (Fig. 5b), $K_{ADP} > 1.3$ mM.

Isotope trapping

Isotope trapping was carried out at room temperature (23–26°C) at pH 8.5 in 50 mM-Tris/HCl buffer containing 50 mM-KCl and 5 mM free Mg^{2+} . Virtually all of the [³²P]phosphoenolpyruvate bound to the enzyme (calculated by using a binding constant of 65 μ M) was trapped as [γ -³²P]ATP, but no trapping of [8-¹⁴C]ATP was detected (Table 2). If ADP were bound by the enzyme in a rate-limiting step, significant trapping should have been observed, provided that the dissociation constant for ADP is less than 20 mM. Further, the relatively low ionic strength of

Fig. 5. Binding of $[\text{P}^{32}]$ phosphoenolpyruvate and $[\text{C}^{14}]$ ADP to pyruvate kinase

Measurements were made by using a rapid-equilibrium-dialysis cell. At intervals, unlabelled substrate was added to give the concentration shown. Mg^{2+} was added with the substrates to maintain the free Mg^{2+} concentration constant. Tetraethylammonium chloride was added to give I 0.2. The abscissa represents 2 ml fractions; 0.5 ml samples were withdrawn and their radioactivities counted. The points illustrated were corrected as described in the text. (a) $3.9 \mu\text{M}$ $[\text{P}^{32}]$ Phosphoenolpyruvate and $27 \mu\text{M}$ pyruvate kinase in 50 mM-Tris/HCl buffer, pH 8.5, containing 50 mM-KCl and 5 mM-free Mg^{2+} . A Scatchard plot is shown inset. (b) $8.6 \mu\text{M}$ $[\text{C}^{14}]$ ADP and $17 \mu\text{M}$ pyruvate kinase in the buffer system described above. At fraction number 50 a further portion of $[\text{C}^{14}]$ ADP was added. The doubling of the radioactivity in the diffusate indicates that the kinetics of diffusion of $[\text{C}^{14}]$ ADP did not change during the experiment.

Table 2. *Isotope-trapping experiments*

A 0.1 ml portion of a 'pulse' solution containing pyruvate kinase, 50 mM-Tris/HCl buffer, pH 8.5, 50 mM-KCl, 5 mM-MgCl₂ and either [³²P]phosphoenolpyruvate or [8-¹⁴C]ADP was rapidly added at room temperature (23–26°C) to 1 ml of a 'chase' solution of unlabelled phosphoenolpyruvate and ADP and sufficient MgCl₂ to give a free Mg²⁺ concentration of 5 mM. After approx. 1 s 1 ml of 1 M-HClO₄ was added, and after 5 min 1 ml of this solution was neutralized with 0.5 ml of 1 M-KHCO₃. The solution was centrifuged to remove KClO₄, ATP, [³²P]phosphoenolpyruvate, [³²P]ATP, [8-¹⁴C]ADP and [8-¹⁴C]ATP were determined as described in the Materials and Methods section. The amount of labelled substrate bound to the enzyme in the 'pulse' solution was calculated by using a binding constant of 65 μM for phosphoenolpyruvate and 2 mM for ADP (see Fig. 5 and the text), and assuming four active sites per enzyme molecule (Cottam *et al.*, 1969).

	Quantity of ATP trapped (nmol)	Pyruvate kinase (active sites) (nmol)	Substrate bound to enzyme (nmol)	Labelled substrate trapped (as % bound)
[³² P]Phosphoenolpyruvate	1.50	4.29	1.37	109
	1.30	4.26	1.37	95
[8- ¹⁴ C]ADP	−0.08	6.2	0.6	0
	−0.09	6.12	0.6	0

the 'pulse' solution (0.09 mol/l) might have been expected to increase any binding of ADP.

Discussion

Interpretation of results

Addition of substrates. A ratio Flux of ATP to ADP/Flux of ATP to phosphoenolpyruvate greater than unity (Fig. 1a) is unequivocal evidence that one pathway for the enzyme reaction involves the addition of phosphoenolpyruvate to the free enzyme in a rate-limiting step. Isotope effects may cause errors in the determination of the fluxes, but with ¹⁴C and ³²P such effects would seem to be far too small to influence the results significantly. The hyperbolic rise in the ratio towards a plateau as the ADP concentration was increased also indicates that the substrates can add in reverse order. The ratio was unaffected by phosphoenolpyruvate concentration, and it follows that (a) the release and addition of ADP from the free enzyme is rapid and/or (b) the ratio $k_{-7}/(k_{-2}+k_{-7})$ is very small (Scheme 2 of Fig. 1 in Britton & Dann, 1978). There is evidence from the isotope-trapping data (see below) that condition (a) applies.

For a random addition and if the phosphoenolpyruvate concentration does not affect the flux ratio, from eqn. (3) of Britton & Dann (1978):

$$\frac{\text{Flux of ATP to ADP}}{\text{Flux of ATP to phosphoenolpyruvate}} = \frac{1 + \alpha[\text{ADP}]}{1 + \beta[\text{ADP}]} \quad (3)$$

where α and β are constants depending on the rate constants. Further, if the addition of ADP is rapid as in Scheme 3 (of Fig. 1 in Britton & Dann, 1978):

$$\alpha = \frac{\phi_A}{\phi_{AB}} - \frac{1}{K_B}$$

where K_B is the dissociation constant of the [E-ADP] binary complex. Assuming $K_B = 2$ mM (see below) and

with the values of ϕ_A and ϕ_{AB} in Table 1, a value of 1.78 mM^{−1} is obtained for α . Line I in Fig. 1(a) represents eqn. (3) with this value and a value of β of 0.588 mM. The latter value was arbitrarily taken to give a flux ratio of 2.1 at an ADP concentration of 2 mM. The theoretical line fits reasonably with the experimental points and yields a plateau value for R_B of 3.04. From eqn. (17) of Britton & Dann (1978) ($\delta = \gamma = 0$ and $R_A = 1$) it follows that at low ADP concentrations at least 67% of the reaction follows the upper pathway in Scheme 3 (of Fig. 1 in Britton & Dann, 1978). At higher ADP concentrations the proportion following the lower pathway increases. For example, at an ADP concentration of 1 mM the proportion following this pathway will lie between 37 and 47%.

An ordered addition with phosphoenolpyruvate adding first to the enzyme would yield a flux ratio independent of phosphoenolpyruvate concentration, as observed (Fig. 1b), but rising linearly with the concentration of ADP. Although the latter condition appeared not to be fulfilled (Fig. 1a), the experimental points show a considerable scatter. Further evidence to exclude an ordered addition may be obtained by calculating the slope of the theoretical line for this mechanism from the steady-state velocity data [eqn. (23) of Britton & Dann, 1978]. From the [3-¹⁴C]pyruvate experiments (see the Results section) and eqn. (24) of Britton & Dann (1978):

$$\frac{Y}{1 + (\phi_A/\phi_{AB})[\text{ADP}]} < 0.008 \quad (4)$$

The ADP concentration was 0.113 mM (Fig. 2a) and, from the values of ϕ_A and ϕ_{AB} (Table 1):

$$Y < 0.01 \quad (5)$$

In eqn. (23) of Britton & Dann (1978), B represents ADP and Q pyruvate. The pyruvate concentration (Fig. 1a) was 40 mM and the $(1 + Y[Q])$ term in the

expression must lie between 1 and 1.4. The latter value represents an extreme upper limit, since the expression for Y contains a [pyruvate] term in the denominator and Y was determined at a relatively low pyruvate concentration. The slope of the line relating the flux ratio to ADP concentration for an ordered addition will thus be approximated by ϕ_A/ϕ_{AB} (broken line II in Fig. 1a). At an ADP concentration of 2mM this line predicts a flux ratio of about 5.5, nearly 3 times greater than the measured value.

The presence of isotope trapping with [^{32}P]-phosphoenolpyruvate (Table 2) provides independent and unequivocal evidence that one pathway involves the addition of phosphoenolpyruvate to the free enzyme in a rate-limiting step. Further, if the addition of phosphoenolpyruvate should be a two-stage process, with a rapid addition and a slow isomerization of the binary complex, the equilibrium for the isomerization must lie well to the right. It also follows that the release of phosphoenolpyruvate from the ternary complex must be a slow step when compared with the conversion of the ternary complex into products. Because of the absence of isotope trapping with [$8\text{-}^{14}\text{C}$]ADP, the method gives no evidence for the existence of the alternative pathway. However, assuming the presence of this pathway, the ADP results indicate that the binding of ADP by the enzyme must be by a rapid step unless the free enzyme has an extremely low affinity for ADP (>20mM).

The particular mechanism of addition of substrates indicated by the flux ratio and isotope-trapping data requires the existence of high-affinity binding sites for phosphoenolpyruvate. Their presence was confirmed by the binding experiments, the somewhat higher dissociation constant in the presence of Mg^{2+} suggesting that phosphoenolpyruvate is bound in the unligated form. Calculation of the dissociation constant from the rate constants for the reaction (see below) shows reasonable agreement with the measured value. In contrast, no binding of ADP ($K_{\text{ADP}} > 1.3\text{ mM}$) could be found in either the presence of Mg^{2+} (5 mM free) or its absence. Thus, when the free enzyme binds ADP first, it appears to do so at a binding site of low affinity. Although such evidence and the isotope-trapping data require that the behaviour of the enzyme during its first turnover is typical, a low-affinity site is also a requirement for the enzyme to obey hyperbolic steady-state velocity kinetics (see below).

Dissociation of products. From the experiments on the incorporation of labelled products into phosphoenolpyruvate the ratio Flux of phosphoenolpyruvate to pyruvate/Flux of phosphoenolpyruvate to ATP (and its reciprocal) must be close to unity even at relatively high pyruvate and ATP concentrations (Figs. 2a and 2b). This on its own does not allow deductions about the order of dissociation of products. However, the product inhibition results

enable the theoretical flux ratios and the incorporation of label into phosphoenolpyruvate to be calculated for ordered dissociations of the products assuming that either ATP or pyruvate dissociates first from the enzyme [see Britton & Dann (1978) and Appendix II]. The calculations indicate that a large incorporation of label into phosphoenolpyruvate should occur (see theoretical lines in Figs. 2a and 2b). Since no incorporation was found, these experiments exclude ordered mechanisms and indicate a random dissociation of products. This assumes that the products inhibit by simple reversal of the enzyme reaction. Inhibition by the formation of dead-end complexes, for example, could interfere with the interpretation. Ainsworth & MacFarlane (1973) proposed the formation of a dead-end complex E-ADP-pyruvate. This introduces a ($\phi_A^{\text{P}}[\text{P}]/[\text{A}]$) term into the steady-state reciprocal velocity equation [where P is pyruvate, A is phosphoenolpyruvate and ϕ_A^{P} is a Dalziel coefficient (Dalziel, 1957)]. In the present work this would increase the apparent value of the coefficient ϕ_{AB}^{P} (Table 1). The term, however, would have to be very large to alter the interpretation of the present data, and measurements of the Michaelis constants for the reaction in the reverse direction (Dyson *et al.*, 1975; Giles *et al.*, 1976) have yielded values of the same order as those calculated from the inhibition data. The rates of exchange at equilibrium (see Appendix I) also show that the enzyme is reversible at pH 8.5.

From the above, the dissociation of one of the products from its binary complex must be rapid. The dissociation of the other product need not be rapid if dissociation of the ternary complex along this alternative pathway is relatively infrequent (Britton & Dann, 1978). However, both ATP and pyruvate approximate to competitive inhibition with respect to phosphoenolpyruvate (Figs. 4a and 4b), which suggests that the dissociation of both products must be relatively rapid.

Ping-Pong mechanism. A Ping-Pong mechanism (Appendix III) with the intermediate formation of a phosphoenzyme is incompatible with the steady-state rate data, since parallel plots should have been obtained in Fig. 3. Such a mechanism can also be excluded by the lack of incorporation of label from [^{14}C]pyruvate into phosphoenolpyruvate. With the values of ϕ_A , ϕ_B and ϕ_{AB}^{P} for pyruvate in Table 1, incorporation of label would have been 44% at 14mM-phosphoenolpyruvate and 85% at 9mM-phosphoenolpyruvate. The ϕ values would therefore have to be very different from those measured to explain the observed lack of incorporation of label.

Calculation of rate constants. Scheme 3 (of Fig. 1 in Britton & Dann, 1978), which is consistent with the observed kinetics, contains ten independent constants, but, since the enzyme approximates to hyperbolic steady-state velocity kinetics, only eight

Fig. 6. Rate constants calculated from the steady-state velocity and isotope data

A is phosphoenolpyruvate, B is ADP, P is ATP and Q is pyruvate. (a) and (b) represent the two extremes, and intermediate values are possible. K_B (Scheme 3 of Fig. 1 in Britton & Dann, 1978) has been assumed to be 2 mM.

independent equations can be set up for their determination. To obtain a solution (see Appendix I) K_B has been assumed to be 2 mM and the range of values for the remaining nine constants are shown in Fig. 6. The constants in Fig. 6(b) correspond to the maximum contribution of the pathway in which ADP adds first to the enzyme and those in Fig. 6(a) to the minimum contribution. The dissociation constant for phosphoenolpyruvate binding to the free enzyme is the same throughout (82 μM) and in good agreement with the value (65 μM) found in the binding studies. The rate constants indicate marked positive cooperativity in the binding of substrates. Thus the assumed value of 2 mM for the dissociation constant for ADP binding to the free enzyme is lowered by the addition of phosphoenolpyruvate to 0.266 mM in Fig. 6(a) and to 23.5 μM in Fig. 6(b). Correspondingly the addition of ADP lowers the dissociation constant for phosphoenolpyruvate to 10.9 μM (Fig. 6a) or to 0.96 mM (Fig. 6b). The data in Fig. 6(a) indicate that a maximum of 68% trapping should have been obtained with [^{32}P]phosphoenolpyruvate, whereas 96% should occur with the constants in Fig. 6(b). Thus the rate constants may approximate more closely to those in Fig. 6(b).

A value for K_B substantially less than 2 mM would not be compatible with the binding data and the steady-state velocity would not obey hyperbolic kinetics. The latter excludes the possibility that the enzyme has an abnormally low affinity for ADP

during its first turnover. If K_B is greater than 2 mM, k_{+7} will be increased approximately in proportion to K_B , but the other rate constants will be little affected. If $K_B > 20 \text{ mM}$, the binding of ADP by the enzyme need not be rapid to explain the isotope-trapping data.

Alternative mechanisms. For simplicity it has been assumed in Scheme 3 (of Fig. 1 in Britton & Dann, 1978) that the dissociations of EPQ into EQ and EP are rapid steps. However, the same kinetics would be obtained if these steps were rate-limiting, provided that the dissociations of the binary complexes EQ and EP are rapid. Similarly the same kinetics will be obtained if the complex EAB dissociates directly into EQ and EP by rate-limiting steps. In these cases the rate constants for the addition of substrates will still be in Fig. 6(b).

General discussion

Mechanism of muscle pyruvate kinase. In initial-velocity studies on rabbit muscle pyruvate kinase at pH 8.5 in glycylglycine buffer, Reynard *et al.* (1961) found that the enzyme obeyed hyperbolic steady-state kinetics. ATP was a competitive inhibitor with respect to both phosphoenolpyruvate and ADP. They concluded that the mechanism was rapid random. Ainsworth & MacFarlane (1973) made initial-velocity studies at pH 6.2 in sodium cacodylate buffer at an ionic strength of 0.2 mol/l while the free Mg^{2+} was

maintained constant and also found that the enzyme conformed to hyperbolic kinetics. They concluded that the mechanism was rapid random, since the products gave the appropriate competitive inhibition patterns, apart from pyruvate, which showed non-competitive inhibition with respect to ADP. The last was attributed to the formation of a dead-end complex E-ADP-pyruvate. In contrast, Melchior (1965), in a careful study at pH 7.0 and I 0.5, found that the initial velocities did not obey hyperbolic kinetics at high ADP concentrations and suggested that the reaction was random but not rapid. Robinson & Rose (1972) also suggested, from experiments on the incorporation of ^3H from water into phosphoenolpyruvate, that addition of substrates might not be rapid, but there are alternative interpretations of their findings.

The intersection close to or on the abscissa of the reciprocal plots of velocity against substrate concentrations in the experiments of Reynard *et al.* (1961) and Ainsworth & MacFarlane (1973), together with the assumption of a rapid random mechanism, imply that the binding of one substrate does not greatly affect the binding of the other. Our steady-state data would lead to the same conclusions if the addition is assumed to be rapid and random. Reynard *et al.* (1961), Mildvan & Cohn (1966) and James *et al.* (1973) found that the free enzyme bound phosphoenolpyruvate with a dissociation constant similar to the K_m , as required by the assumption of a rapid random mechanism. However, Reynard *et al.* (1961) found little binding of ADP and were unable to determine a dissociation constant, although they concluded that it must lie within an order of magnitude of the K_m . In contrast, Mildvan & Cohn (1966) found a dissociation constant for ADP of 0.068–0.2 mM at pH 7.5 from magnetic-resonance measurements and protection studies with mercuribenzoate.

Giles *et al.* (1976) have described measurement of equilibrium exchanges between ATP and phosphoenolpyruvate, ATP and ADP and pyruvate and phosphoenolpyruvate at pH 7.4 and 25°C. All the exchange rates were the same within the statistical error, apart from that for the exchange between phosphoenolpyruvate and pyruvate, which was lower. The exchange rates between phosphoenolpyruvate, ADP and ATP are consistent with our own findings at pH 8.5. However, the maximum ADP concentration used by Giles *et al.* (1976) was only 0.05 mM, and thus, on the basis of the steady-state data for the forward reaction reported in the present paper, such measurement will not distinguish between an ordered or random addition of substrates. The slower exchanges between phosphoenolpyruvate and pyruvate are not consistent with our finding, since they imply that the ratio Flux of phosphoenolpyruvate to ATP/Flux of phosphoenolpyruvate to pyruvate must be substantially greater than unity at ATP

concentrations of 1 mM and 7.65 mM. Measurements of this ratio at pH 7.4 may therefore be valuable.

Our study at pH 8.5 has shown by two unequivocal methods that one pathway for the enzyme involves phosphoenolpyruvate adding first to the enzyme in a rate-limiting step. The substrates can also add in alternative order (although this pathway is a relatively minor one at low ADP concentrations), and there is strong evidence that the dissociation of products is rapid and random (Fig. 6). With the rate constants indicated in Fig. 6, the enzyme will obey hyperbolic kinetics, except at very high ADP concentrations. This mechanism predicts non-competitive inhibition by both pyruvate and ATP with ADP as the varied substrate [eqn. (1) in Appendix I], whereas both Reynard *et al.* (1961) and Ainsworth & MacFarlane (1973) reported competitive inhibition between ATP and ADP. Exact comparisons cannot be made, but both small variations in the concentration of free Mg^{2+} and the non-linearities predicted by eqn. (1) (in Appendix I) may have minimized the non-competitive element in these experiments. Our binding studies have confirmed the binding of phosphoenolpyruvate and have shown that it is probably the unligated species that is bound to the enzyme. However, our observations indicate that the dissociation constant (65 μM) is larger than the K_m determined under the same conditions (32.5 μM), as required if the addition of phosphoenolpyruvate to the free enzyme is a rate-limiting step. We have been unable to demonstrate binding of ADP either in the presence or in the absence of Mg^{2+} , despite a K_m for ADP of 170 μM , and we are unable to confirm the findings obtained by Mildvan & Cohn (1966). Although binding of ADP would have been expected if the mechanism were rapid and random, a large dissociation constant is a necessary requirement if a non-rapid random mechanism is to exhibit hyperbolic steady-state kinetics. Our binding data are therefore in complete accord with our kinetic data, and lead to the conclusion that the binding of either substrate increases the affinity of the enzyme for the other substrate by a very large factor. With the rate constants indicated in Fig. 6 the increase is 7.5–8.5 times, and the increase may be larger because a K_{ADP} of 2 mM represents a minimum value. Such large changes clearly have considerable implications for the molecular mechanism of the enzyme.

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APPENDIX I

Kinetics of a Branched Pathway, Conditions for Approximating to Hyperbolic Steady-State Velocity Kinetics and Calculation of Rate Constants and Flux Ratios

For Scheme 3 (of Fig. 1 in Britton & Dann, 1978), the velocity v of the reaction in the absence of Q is given by the expression:

$$\frac{1}{v} = \frac{1}{(E_T)} \left[\frac{1}{k_{+3}} + \frac{k_{-7}}{k_{+3}k_{-2}[D]} + \frac{(k_{+3} + k_{-7})k_{+2} + k_{-1}(k_{-2} + k_{+3} + k_{-7})/K_B}{k_{+1}k_{+2}k_{+3}[A][D]} + \frac{(k_{+3} + k_{-7})[P]}{k_{+1}k_{+3}[A][D]K_P} \right. \\ \left. + \frac{(k_{+3} + k_{-7})[B]}{k_{+1}k_{+3}[A]K_B} + \frac{(k_{-2} + k_{+3} + k_{-7})}{k_{+2}k_{+3}[B][D]} + \frac{k_{-1}(k_{-2} + k_{+3} + k_{-7})}{k_{+1}k_{+2}k_{+3}[A][B][D]} \left(1 + \frac{[P]}{K_P} \right) \right] \quad (1)$$

where

$$[D] = 1 + (k_{-7}/k_{-2}) + (k_{-7}k_{+2}/k_{-1}k_{-2}[B]) \quad (2)$$

and (E_T) represents the amount of enzyme.

Also:

$$k_{+1}k_{+2}k_{+3}K_{PQ}/k_{-1}k_{-2}k_{-3} = K_{eq.} \quad (3)$$

and

$$k_{+1}k_{+2}k_{-7}K_B = k_{-1}k_{-2}k_{+7} \quad (4)$$

where $K_{eq.}$ is the equilibrium constant for the reaction.

Provided that the $[B]/[A]$ term is small in eqn. (1) and that the $[B]$ term is small in eqn. (2), Scheme 3 (of Fig. 1 in Britton & Dann, 1978) will approximate to hyperbolic steady-state velocity kinetics, as

observed, and the coefficients can be related to the ϕ values [Table 1 of the main paper (Dann & Britton, 1978)] and the inhibition data as follows:

$$(1/k_{+3})(1 + k_{-7}/k_{-2}[D]) = \phi_0 \quad (5)$$

$$(k_{+3} + k_{-7})/k_{+1}k_{+3}[D] = \phi_A - \phi_{AB}/K_B \quad (6)$$

$$(k_{-2} + k_{+3} + k_{-7})/k_{+3}k_{+2}[D] = \phi_B \quad (7)$$

$$k_{-1}(k_{-2} + k_{+3} + k_{-7})/k_{+1}k_{+2}k_{+3}[D] = \phi_{AB} \quad (8)$$

$$K_P = [\phi_A + \phi_{AB}(1/[B] - 1/K_B)]/m_P \quad (9)$$

where m_P is the slope of the replot for ATP [Fig. 4(b) and Table 1 of the main paper (Dann & Britton, 1978)]. Since a similar expression to eqn. (1) holds for the reaction in the presence of Q but in the absence of P:

$$K_Q = [\phi_A + \phi_{AB}(1/[B] - 1/K_B)]/m_Q \quad (10)$$

where m_Q is the slope of the replot for pyruvate [Fig. 4(a) and Table 1 of the main paper (Dann & Britton, 1978)]. The values of K_P and K_Q given in Fig. 6 of the main paper (Dann & Britton, 1978) were calculated from these values and assuming $K_B = 2$ mM.

Since [B] ([ADP]) was $100\mu\text{M}$ and $50\mu\text{M}$ respectively, K_P and K_Q will be affected only negligibly if K_B has a larger value. From eqn. (5) (Britton & Dann, 1978):

$$k_{+2}k_{-7}/k_{-1}(k_{-2}+k_{-7})=\beta \quad (11)$$

At chemical equilibrium the flux of A to P (Britton, 1966) is given by the expression:

$$[\text{Flux of A to P}]^{-1} = \frac{1}{(E)} \left[\frac{(k_{+3}+k_{-7})}{k_{+1}k_{+3}[A][D]} + \frac{k_{-1}(k_{-2}+k_{-7}+k_{+3})}{k_{+1}k_{+2}k_{+3}[A][B][D]} \right] \quad (12)$$

and

$$\frac{1}{(E)} = \frac{1}{(E_T)} \left[1 + \frac{k_{+1}[A]}{k_{-1}} + \frac{[B]}{K_B} + \frac{[P]}{K_P} + \frac{[Q]}{K_Q} + \frac{k_{+1}k_{+2}}{k_{-1}k_{-2}} \left(1 + \frac{k_{+3}}{k_{-3}} \right) [A][B] \right] \quad (13)$$

where (E) represents the amount of free enzyme.

The coefficients in eqn. (12) are given by eqns. (6) and (7). Further, from eqns. (7) and (8) $k_{+1}/k_{-1} = \phi_B/\phi_{AB}$, and, if a value for K_B of 2 mM is assumed, all of the coefficients except the last in eqn. (13) are known. Defining this coefficient as ϕ_E :

$$\phi_E = k_{+1}k_{+2}(1+k_{+3}/k_{-3})/k_{-1}k_{-2} \quad (14)$$

in three experiments the rate of exchange between phosphoenolpyruvate and ATP was measured with enzyme that had been freshly assayed. With these results and the values of ϕ in Table 1 of the main paper (Dann & Britton, 1978) a mean value ($\pm$ S.E.M.) for ϕ_E of 622 ± 87 was obtained.

Eqns. (3)–(11) and (14) relate the rate and equilibrium constants to measured parameters [see Table 1 and Results section of the main paper (Dann & Britton, 1978)]. The range of solutions in Fig. 6 of the main paper (Dann & Britton, 1978) was obtained as follows. K_B was assumed to be 2 mM, and to a first approximation $k_{+3} = \phi_0^{-1}$. From eqns. (7), (8) and (14):

$$k_{-2} \geq k_{+1}k_{+2}/k_{-1}\phi_E \geq 1/\phi_{AB}\phi_E \quad (15)$$

With the minimum value for k_{-2} given by eqn. (15) and the approximate value for k_{+3} , a quadratic equation in k_{-7} was derived from eqns. (6), (8) and (11). These values for k_{-2} , k_{-7} and k_{+3} were then used to derive solutions for the other rate constants, assuming $[D] = 1$. A revised value for k_{-2} was then taken, based on eqn. (15). Finally, a value for k_{+3} calculated from eqn. (5), and similarly values for the other rate constants, were obtained by inserting values for the rate constants in the expression for $[D]$. Iterations of the whole procedure led to the solution given in Fig. 6(b) of the main paper (Dann & Britton, 1978). To obtain the solutions shown in Fig. 6(a) of the

main paper (Dann & Britton, 1978), a similar procedure was used, but in this case the quadratic equation in k_{-7} does not contain k_{-2} .

Since $K_B > 1.3\text{ mM}$ and in the initial-velocity experiments the maximum ADP concentration (substrate B in Scheme 3 of Fig. 1 in Britton & Dann, 1978) was 0.2 mM, it follows from eqn. (1) that, even at the maximum ADP concentration, the $[B]/[A]$ term in eqn. (1) will be less than one-fifth of the $1/[A]$ term. The $1/[A]$ term becomes most important at low phosphoenolpyruvate concentrations (substrate A), but, even then, with the values from Table 1 of the main paper (Dann & Britton, 1978), the $[B]/[A]$ term contributes less than 5% to the velocity. The contribution of the $[B]/[A]$ term is thus negligible, as is required if Scheme 3 (of Fig. 1 in Britton & Dann, 1978) is to obey hyperbolic steady-state velocity. The $[B]$ term in the expression for $[D]$ (eqn. 2) should also be small. With the values in Figs. 6(a) and 6(b) of the main paper (Dann & Britton, 1978), the maximum contribution of the B term is about 10%. Further, the contributions of the $[B]/[A]$ term and the $[D]$ term will be in opposite directions and will tend to cancel each other.

If the $[B]$ term were the major term in the expression for $[D]$, the identification of the ϕ terms with the coefficients in eqn. (1) would be incorrect. This possibility can be excluded on the following grounds. (1) From eqn. (10) of Britton & Dann (1978), and since $R_B = 3.04$, $k_{-7}/k_{-2} < 0.5$. The $[B]$ term in the expression for $[D]$ could therefore only be large if $k_{+2}[B] > k_{-1}$, this corresponding to measurements at near-saturating concentrations of B. (2) Terms containing $[B]^{-2}$ would appear in eqn. (1) and the plots of v^{-1} versus $[B]^{-1}$ should be non-linear. (3) Even if the $[B]^{-2}$ terms in eqn. (1) should be small, and, provided that $K_B > 1.3\text{ mM}$, ϕ_{AB} should be greater than ϕ_A , contrary to observation.

Flux Ratio for an Ordered Dissociation of Products

If the addition of substrates is as in Scheme 3 (of Fig. 1 in Britton & Dann, 1978), but the dissociation of products is ordered with P dissociating first from the ternary complex, then with the rate constants as defined in Schemes 2 and 3 (of Fig. 1 in Britton & Dann, 1978):

$$\frac{\text{Flux of A to P}}{\text{Flux of A to Q}} = 1 + \frac{k_{-4}Z[P]}{k_{+5}(Z+k_{+4})} \quad (16)$$

and

$$Z = \frac{k_{-3}(k_{-1}(k_{-2}+k_{-7})+k_{-7}k_{+2}[B])}{k_{-1}(k_{-2}+k_{-7}+k_{+3})+(k_{-7}+k_{+3})k_{+3}[B]} \quad (17)$$

Further:

$$\phi_{AB} = k_{-1}[(k_{-3}+k_{+4})(k_{-2}+k_{-7})+k_{+3}k_{+4}]/k_{+3}k_{+4}[D] \quad (18)$$

$$\phi_{AB}^P = k_{-1}k_{-3}k_{-4}(k_{-2}+k_{-7})/k_{+3}k_{+4}k_{+5}[D] \quad (19)$$

and

$$\phi_A = \{k_{+2}[k_{-2}(k_{-3} + k_{+4}) + k_{+3}k_{+4}] + k_{-1}[(k_{-2} + k_{-7})(k_{-3} + k_{+4}) + k_{+3}k_{+4}]/K_B\}/k_{+3}k_{+4}[D] \quad (20)$$

Inspection of eqns. (16)–(20) indicates that the coefficient for [P] in eqn. (16) will be intermediate in value between those given by eqn. (28) and eqn. (29) of Britton & Dann (1978).

APPENDIX II

Calculation of the Incorporation of Radioactivity into a Substrate from a Labelled Product

Consider the experiment illustrated in Fig. 2(b) of the main paper (Dann & Britton, 1978), in which the reaction was carried out in the presence of labelled ATP. Let A be phosphoenolpyruvate, B be ADP, P be ATP and Q be pyruvate, and let:

$$r = \frac{\text{Flux of A to P}}{\text{Flux of A to Q}} \quad (1)$$

Then from eqn. (37) of Britton & Dann (1978):

$$\text{Flux of P to A} = (r-1) \times \text{Net flux of A to Q}$$

If N is total radioactivity, n_a is radioactivity in A, n_g is radioactivity in glucose 6-phosphate and $N - (n_a + n_g)$ is radioactivity in ATP, then:

$$dn_a = \frac{n_a}{[A]} d[A] - (r-1) \frac{N - (n_a + n_g)}{[Q]} d[A] \quad (2)$$

and

$$dn_g = - \frac{N - (n_a + n_g)}{[Q]} d[A] \quad (3)$$

If the dissociation of products is ordered with P dissociating first from the ternary complex, then the minimum value of r is given by eqn. (28) of Britton & Dann (1978). This corresponds to an ordered addition, with A adding first to the enzyme. The maximum value of r is given by eqn. (29) of Britton & Dann (1978), corresponding to an ordered addition with the substrate last to add to the enzyme or to a rapid random addition. A random addition of

References

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substrates of the type shown in Scheme 3 (of Fig. 1 in Britton & Dann, 1978) gives an intermediate value for r (see Appendix I).

To solve eqns. (2) and (3), eqns. (28) and (29) of Britton & Dann (1978) and the values of ϕ given in Table 1 of the main paper (Dann & Britton, 1978) were used to determine r . Eqns. (2) and (3) were then solved numerically by stepwise integration. In the initial calculations iterative procedure was used to determine dn_a and dn_g , but with the particular conditions iteration was found to be unnecessary.

In the experiment illustrated in Fig. 4(a) of the main paper (Dann & Britton, 1978) the reaction was carried out in the presence of labelled pyruvate. In this case radioactivity can only enter phosphoenolpyruvate, and $n_g = 0$. Eqn. (2) can be integrated to give:

$$\frac{n_a}{N} = \frac{k[A]}{\exp(-k[A])} \left[\ln \frac{[A_0]}{[A]} - \left(k([A_0] - [A]) - \frac{k^2([A_0]^2 - [A]^2)}{2.2!} + \frac{k^3([A_0]^3 - [A]^3)}{3.3!} \dots \right) \right] \quad (4)$$

where $[A_0]$ is the initial concentration of A, and k is $(1-r)/r[P]$. r was calculated as above but assuming that P is pyruvate.

References

- Britton, H. G. & Dann, L. G. (1978) *Biochem. J.* **169**, 29–37
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APPENDIX III

Flux Ratios for a Ping-Pong Mechanism

From eqn. (31) of Britton & Dann (1978) and the values in Table 1 of the main paper (Dann & Britton, 1978) at 2 mM-ADP:

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} > 1.75$$

The measured values for this ratio therefore fall within the allowed range. However, in the expression for the ratio Flux of A to Q/Flux of A to P [eqn. (32) of Britton & Dann (1978)] $(\phi_A^Q + \phi_{AB}^Q/[B])$ corresponds

to the slope replot m_Q . With this value and the value for ϕ_A [Table 1 of the main paper (Dann & Britton, 1978)] the calculated incorporation of label into phosphoenolpyruvate is very large, and a Ping-Pong mechanism is therefore excluded.

References

- Britton, H. G. & Dann, L. G. (1978) *Biochem. J.* **169**, 29–37
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