The mechanism of rabbit muscle phosphofructokinase at pH8

Stephen MERRY* and Hubert G. BRITTON

Department of Physiology, St. Mary's Hospital Medical School, London W2 1PG, U.K.

(Received 21 May 1984/Accepted 1 October 1984)

1. The mechanism of rabbit muscle phosphofructokinase was investigated by measurement of fluxes, isotope trapping and steady-state velocities at pH8 in triethanolamine/HCl buffer with 4mM free Mg^{2+} . Most observations were made at 10.2. 2. The ratio Flux of fructose 1,6-bisphosphate→fructose 6-phosphate/Flux of fructose 1,6-bisphosphate→ATP at zero ATP concentration increased hyperbolically from unity to about 3.2 as the concentration of fructose 6-phosphate was increased. Similarly, the ratio Flux of fructose 1,6-bisphosphate→ATP/Flux of fructose 1,6-bisphosphate→fructose 6-phosphate at zero fructose 6-phosphate concentration increased from unity to about 1.4 as the concentration of ATP was increased. The addition of substrates must therefore be random, whatever the other aspects of the reaction. Further, from the plateau values of the ratios, it follows that the substrates dissociate very infrequently from the ternary complex and that at a low substrate concentration 72% of the reaction follows the pathway in which ATP adds first to the enzyme. 3. Isotope-trapping studies with [^{32}P]ATP confirmed that ATP can bind first to the enzyme in rate-limiting step and that dissociation of ATP from the ternary complex is slow in relation to the forward reaction. No isotope trapping of [U- ^{14}C]fructose 6-phosphate could be demonstrated. 4. The ratios Flux of ATP→fructose 1,6-bisphosphate/Flux of ATP→ADP measured at zero ADP concentration and the reciprocal of the ratio measured at zero fructose 1,6-bisphosphate concentration did not differ significantly from unity. Calculated values for these ratios based on the kinetics of the reverse reaction and assuming ordered dissociations of products or a ping-pong mechanism gave values very significantly greater than unity. These findings exclude an ordered dissociation or a substantial contribution from a ping-pong mechanism, and it is concluded that the reaction is sequential and that dissociation of products is random. 5. Rate constants were calculated for the steps in the enzyme reaction. The results indicate a considerable degree of co-operativity in the binding between the two substrates. 6. The observations on phosphofructokinase are discussed in relation to methods of measurement and interpretation of flux ratios and in relation to the mechanism of other kinase enzymes.

Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyses the transfer of the terminal phosphate of ATP to the C-1 hydroxy group of Fru6P to produce Fru(1,6) P_2 and ADP. It is an important glycolytic enzyme, particularly because it is subject to metabolic control and its velocity is modified by a

number of metabolites, including ATP, AMP, citrate and Fru(1,6) P_2 (Bloxham & Lardy, 1973). The rabbit muscle enzyme is also activated by Mg^{2+} , K^+ , low concentrations of NH_4^+ and dithiothreitol (Paetkau & Lardy, 1967).

The skeletal-muscle enzyme has been the subject of a number of kinetic investigations, but with differing conclusions about the mechanism. Uyeda (1970) favoured a ping-pong mechanism involving a phosphorylated enzyme intermediate. Hanson (1970) reported, from steady-state velocity measurements, a sequential mechanism with an ordered addition, with ATP first to add to the

Abbreviations used: Fru6P, fructose 6-phosphate; Fru(1,6) P_2 , fructose 1,6-bisphosphate.

* Present address: University of Glasgow Department of Oncology, 1 Horselethill Road, Glasgow G12 9LY, Scotland, U.K.

enzyme and an ordered dissociation with ADP last to leave the enzyme. However, Kee & Griffin (1972) and Hanson *et al.* (1973) concluded that the mechanism was rapid random. Bar-Tana & Cleland (1974) reported that the mechanism was non-rapid random in the forward direction on the basis of non-linear reciprocal plots and rapid random in the reverse direction. In this latter work, however, the concentration of free Mg^{2+} was not maintained constant, which, in itself, will lead to non-linear plots. With the sheep heart enzyme, with fructose 6-sulphate as an alternative substrate, Martensen & Mansour (1976) concluded that the mechanism is ordered, with ATP adding to the free enzyme. All of the above investigations were carried out in alkaline solution (pH 8–8.5), where the enzyme exhibits Michaelis–Menten kinetics with respect to Fru6P and ATP. In a review Uyeda (1979) concludes that the mechanism has not been unequivocally determined as random or ordered.

The problems inherent in the interpretation of steady-state velocity data with respect to mechanism appear to be responsible for the variety of conclusions that have been reached. The present paper describes the results of investigation of the mechanism of rabbit muscle phosphofructokinase with the use of a variety of techniques including flux-ratio measurements with labelled substrates (Britton, 1966; Britton & Dann, 1978) isotope trapping (Rose *et al.*, 1974) in addition to initial-velocity studies. A short note has already been published (Merry & Britton, 1981).

Materials and methods

Materials

Substrates and enzymes were obtained from Boehringer Corp. (London W.5, U.K.), ATP, ADP, Fru6P, NADH and NADP⁺ as sodium salts, and Fru(1,6)P₂ and phosphoenolpyruvate as cyclohexylammonium salts. 2,5-Diphenyloxazole was obtained from Packard Instruments (Wembley, Middx., U.K.), 1,4-bis-(5-phenyloxazol-2-yl)-benzene from Koch-Light Laboratories (Colnbrook, Bucks., U.K.), Triton X-100 from Rohm and Haas (Croydon, Surrey, U.K.) and toluene from Fisons Scientific Apparatus (Loughborough, Leics., U.K.). All other chemicals were reagent grade or better and were obtained from BDH Chemicals (Poole, Dorset, U.K.).

[γ -³²P]ATP (sodium or triethylammonium salt), [8-¹⁴C]ADP (ammonium salt) and D-[U-¹⁴C]-Fru6P (potassium salt) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). To prepare [1-³²P]Fru(1,6)P₂, [γ -³²P]ATP (2 μ l; 5 μ Ci; 1.7 nmol; triethylammonium salt) and 2 μ l of a solution of phosphofructokinase [10 mg/ml in 3.2 M (NH₄)₂SO₄ diluted 1:16 with water] were

added to a solution (146 μ l) containing 0.07 mM-ATP, 0.68 mM-Fru(1,6)P₂, 51 mM-KCl, 6.8 mM-MgCl₂ and 34 mM-triethanolamine/HCl buffer, pH 8, and the reaction was left to proceed for 1 h at room temperature (21–24°C). To determine the radiochemical purity of the product, 5 μ l of the solution was added to 10 μ l of ethanol, and 5 μ l of this solution was applied as a 1.5 cm strip on a PEI-cellulose (polyethyleneimine-cellulose) thin-layer plate (Anderman and Co., East Molesey, Surrey, U.K.). Chromatography with 0.6 M-ammonium formate (adjusted to pH 3 with formic acid) and scanning (Packard model 7201 radiochromatogram scanner) showed that at least 90% of the radioactivity migrated as Fru(1,6)P₂. The radiochemical purity of [γ -³²P]ATP was determined as described by Rubio *et al.* (1979). [8-¹⁴C]ADP was assayed for radiochemical purity by chromatography before and after conversion into ATP with a reaction mixture containing pyruvate kinase. Chromatography was carried out by using the methods described for Fru(1,6)P₂. At least 95% of the radioactivity behaved as ADP in the first chromatogram and at least 95% of the radioactivity behaved as ATP in the second chromatogram.

The radiochemical purity of [U-¹⁴C]Fru6P was assayed by a method analogous to that described for [8-¹⁴C]ADP. In this case chromatography was carried out before and after conversion into Fru(1,6)P₂ with phosphofructokinase. At least 95% of the radioactivity behaved as Fru6P in the first chromatogram and at least 95% of the radioactivity behaved as Fru(1,6)P₂ in the second chromatogram.

To remove ATP from phosphofructokinase, the enzyme suspension [10 mg/ml in 3.2 M (NH₄)₂SO₄] was centrifuged, and the precipitate was dissolved in its original volume in a medium containing 50 mM-KCl, 6 mM-(NH₄)₂SO₄, 1 mM-dithiothreitol, 4 mM-MgCl₂ and 30 mM-triethanolamine/HCl buffer, pH 8. The solution was then treated for 3 min at room temperature (approx. 22°C) with acid-washed animal charcoal (10 $\times$ weight of enzyme) and the charcoal was removed by brief centrifugation. The A_{280}/A_{260} ratio was >1.68:1, indicating effective removal of ATP (Parmeggiani *et al.*, 1966). The enzyme was used within 45 min of preparation.

Methods

General. Radioactive compounds were separated on PEI-cellulose thin-layer plates as described above. The chromatograms were scanned and the radioactivity was determined from the areas under the peaks or by liquid-scintillation counting of areas cut from the chromatogram (Dann & Britton, 1978).

Mg^{2+} stability constants for Fru6P, glucose 6-

phosphate, Fru(1,6) P_2 , NAD⁺ and NADP⁺ were determined the method of Burton (1959) under the conditions used in the flux ratio and steady-state velocity experiments. The stability constant of Fru(1,6) P_2 was calculated assuming that 1 Mg²⁺ ion binds to this anion, as assumed for glucose 1,6-bisphosphate (Ray & Roscelli, 1966). The stability constants for ATP and ADP (Belaich & Sari, 1969) and phosphoenolpyruvate (Wold & Ballou, 1957) were taken from the literature.

Fru(1,6) P_2 and Fru6P were assayed spectrophotometrically by using enzyme reactions coupled to the oxidation of NADH.

'Pulse-chase' experiments. [γ -³²P]ATP trapping was carried out with a 'pulse' solution containing (final concentrations) phosphofructokinase (1.6 mg/ml), 50 mM-KCl, 6 mM-(NH₄)₂SO₄, 1 mM-dithiothreitol, MgCl₂ to give 4 mM free Mg²⁺ and 30 mM-triethanolamine/HCl buffer, pH 8 (I/0.08). [γ -³²P]ATP was added, and the mixture was incubated at room temperature for 2 s. A sample (100 μ l) was then rapidly added to 0.9 ml of rapidly stirred 'chase' solution at room temperature (20–22°C), which contained 100 mM-ATP, 10 mM-Fru6P (except where indicated), 50 mM-KCl, 6 mM-(NH₄)₂SO₄, 1 mM-dithiothreitol, 107 mM-MgCl₂ (4 mM free Mg²⁺) and 30 mM-triethanolamine/HCl buffer, pH 8 (I/0.54). After approx. 2 s 1 ml of 1 M-HClO₄ was added, and after 5 min 2 ml of 0.5 M-KHCO₃ was added. Precipitated KClO₄ was removed by centrifugation, and 5 μ l of the supernatant was chromatographed and the chromatogram scanned as above. Less than 2% of the total radioactivity was present in the KClO₄ precipitate. ATP present in the phosphofructokinase was determined by adding [U-¹⁴C]Fru6P (43–233 μ M) to the 'pulse' solution in place of [³²P]ATP. After 2 s the solution (100 μ l) was treated with HClO₄ followed by KHCO₃, and then chromatographed and the chromatogram scanned as above. The ATP content, which was relatively constant in different batches of the enzyme (16.1 nmol of enzyme), was used to correct the specific radioactivity of the ATP in the 'pulse' solution.

Two procedures were used for [U-¹⁴C]Fru6P trapping. The first was similar to that used for the trapping of [³²P]ATP. Non-radioactive Fru6P (48 μ M) was added to the 'pulse' solution, and the mixture was incubated for 5 min to convert ATP in the enzyme preparation into ADP. Then [U-¹⁴C]Fru6P (42 μ M) was added, and after 2 s 100 μ l of the mixture was added to 0.9 ml of 'chase' solution modified to contain 100 mM-Fru6P, 10 mM-ATP and 41 mM-MgCl₂ (4 mM free Mg²⁺) (I/0.3). The second procedure was used at higher concentrations of enzyme (5–10 mg/ml) to conserve materials. The total volume of 'pulse' solution was decreased to 42–44 μ l, and 400 μ l of 'chase' solution

was added to the solution with rapid stirring. After 2 s 1 ml of ethanol was added, and 5 μ l of the mixture was immediately chromatographed as above. In most of these latter experiments, the phosphofructokinase had been treated to remove ATP and the initial step of addition of unlabelled Fru6P was omitted. However, in some experiments at the highest concentration (10 mg/ml) the enzyme was untreated. Unlabelled Fru6P (364 μ M) was then added to the 'pulse' solution, which was incubated for 5 min before the addition of [U-¹⁴C]-Fru6P.

Steady-state velocity and flux measurements. All experiments were carried out at 30°C in 30 mM-triethanolamine/HCl buffer, pH 8, 50 mM-KCl, 6 mM-(NH₄)₂SO₄ and 1 mM-dithiothreitol. MgCl₂ was added to give free Mg²⁺ concentration of 4 mM, and tetraethylammonium chloride was added to give an ionic strength of 0.2.

Steady-state velocities were measured spectrophotometrically in a volume of 3 ml with 10 mm pathlength (SP 1800 spectrophotometer; Pye-Unicam, Cambridge, U.K.). In the direction of Fru(1,6) P_2 synthesis the reaction was coupled to NADH oxidation by the addition of (final concentrations) phosphoenolpyruvate (0.3 mM), NADH (0.3 mM), pyruvate kinase (10 μ g/ml) and lactate dehydrogenase (10 μ g/ml). Phosphofructokinase (0.1–0.28 μ g/ml) was added to start the reaction. In the direction of ATP synthesis the reaction was coupled to NADPH formation by the addition of (final concentrations) NADP⁺ (0.33 mM), phosphoglucose isomerase (10 μ g/ml) and glucose-6-phosphate dehydrogenase (10 μ g/ml), and the reaction was started by the addition of phosphofructokinase (17 μ g/ml). The quantity of coupling enzymes was sufficient for the rate to be independent of their quantity both at high and low substrate concentrations. The unweighted data were fitted to hyperbolic kinetics with the computer program of Cleland (1967).

The ratio Flux of Fru(1,6) P_2 →Fru6P/Flux of Fru(1,6) P_2 →ATP was determined from the incorporation of label into Fru(1,6) P_2 from [U-¹⁴C]-Fru6P while the reaction proceeded in the direction of ATP synthesis (Britton & Dann, 1978). Reaction conditions were identical with those used in the initial-velocity experiments. Fru(1,6) P_2 and ADP concentrations were 5 mM and 1 mM respectively. The [U-¹⁴C]Fru6P concentration was varied in the range 0.1–2 mM, and ATP was maintained at zero by the addition of (final concentrations) glucose (5 mM), NADP⁺ (0.33 mM), hexokinase (10 μ g/ml) and glucose-6-phosphate dehydrogenase (10 μ g/ml). The reaction was started by the addition of phosphofructokinase (0.5 μ g/ml), and the reaction was monitored by the increase in absorbance at 340 nm. Samples (5 μ l)

were taken at intervals of approx. 50 min, added to ethanol (10 μ l) and 5 μ l of the mixture was chromatographed as above. The appropriate areas of the chromatogram were identified by scanning, and the distribution of label was determined by liquid-scintillation counting. Less than 0.15 mM-Fru(1,6) P_2 was consumed during the course of each experiment. Results were corrected for the blank values obtained by carrying out the reaction in the absence of phosphofructokinase. The extent of the reverse reaction (see the Results section and Appendix 1) was determined by using unlabelled Fru6P and by adding [γ - 32 P]ATP (triethylammonium salt) (4–17 nM) shortly after the addition of phosphofructokinase, when the reaction had reached a steady state.

The ratio Flux of Fru(1,6) P_2 →ATP/Flux of Fru(1,6) P_2 →Fru6P was determined in an analogous manner to its reciprocal. Fru(1,6) P_2 and ADP concentrations were 5 mM and 1 mM respectively. The [γ - 32 P]ATP concentration was varied between 0.05 and 1.25 mM, and Fru6P was maintained at zero by the addition of (final concentrations) NADP⁺ (0.33 mM), phosphoglucose isomerase (9.9 μ g/ml) and glucose-6-phosphate dehydrogenase (10 μ g/ml). The reaction was started by the addition of phosphofructokinase (0.24 μ g/ml) and the increase in absorbance at 340 nm was monitored. Samples were chromatographed and the distribution of label was determined as in the measurement of the reciprocal ratio. Less than 0.15 mM-Fru(1,6) P_2 was consumed during the course of each measurement. Data were corrected for blanks obtained by carrying out the reaction in the absence of phosphofructokinase. The extent of the reverse reaction was determined by using unlabelled ATP and adding [U- 14 C]Fru6P (590 nmol) to the reaction mixture when the steady state had been reached.

The ratio Flux of ATP→ADP/Flux of ATP→Fru(1,6) P_2 was determined from the incorporation of label from [8- 14 C]ADP into ATP while the reaction was proceeding in the direction of FDP synthesis (Britton & Dann, 1978). The conditions were as in the initial-velocity experiments. Fru6P and ATP concentrations were each 5 mM. The [8- 14 C]ADP concentration was varied between 0.24 and 2.28 mM, and the total volume was 840 μ l. The reaction was started by the addition of phosphofructokinase. At 0 and 24 min 200 μ l portions of the mixture were each added to 400 μ l of ethanol, and at 6, 12 and 18 min 100 μ l portions were each added to 200 μ l of ethanol. Samples (5 μ l) of the ethanolic mixtures were chromatographed, and duplicate 100 μ l samples were taken for Fru(1,6) P_2 determination. Duplicate samples (100 μ l) from the portions taken at 0 and 24 min were analysed for ADP. Less than

0.25 mM-Fru6P was consumed during each experiment. The data were corrected for the blanks obtained in the absence of phosphofructokinase.

The ratio Flux of ATP→Fru(1,6) P_2 /Flux of ATP→ADP was determined from the incorporation of label from [1- 32 P]Fru(1,6) P_2 into ATP with the reaction proceeding in the direction of Fru(1,6) P_2 synthesis. In one group of experiments, ATP and Fru6P concentrations were 5 mM and 1 mM respectively, and that of Fru(1,6) P_2 was either 0.05 or 0.5 mM. ADP was continuously reconverted into ATP, and the reaction was monitored at 340 nm by the addition of (final concentrations) phosphoenolpyruvate (1 mM), NADH (0.33 mM), pyruvate kinase (10 μ g/ml) and lactate dehydrogenase (10 μ g/ml). At zero time [1- 32 P]-Fru(1,6) P_2 (100 μ l, prepared as described in the Materials and methods section) was added, immediately followed by phosphofructokinase (0.4 μ g/ml). Samples (5 μ l) were taken at zero time and when approx. 0.15 mM-Fru6P had been consumed. The samples were added to ethanol (10 μ l), and 5 μ l of the mixture was chromatographed and the chromatogram scanned. In a second group of experiments Fru6P was maintained at a very low concentration by generating this substrate, *in situ*, from glucose 6-phosphate and a rate-limiting concentration of phosphoglucose isomerase. Glucose 6-phosphate (1 mM) and at zero time phosphoglucose isomerase (0.04 μ g/ml) were added, followed by phosphofructokinase (10 μ g/ml). Samples (100 μ l) were taken from the reaction mixture at zero time and after the reaction had consumed approx. 0.075 mM- and 0.15 mM-glucose 6-phosphate. They were each added to ethanol (400 μ l) and assayed for [32 P]ATP and Fru6P as described above.

Results and discussion

Stability constants

Where literature values of Mg²⁺ stability constants at the appropriate temperature, pH and ionic strength were not available, they were determined under the conditions of the flux and steady-state velocity measurements. The results obtained were 0.092, 0.153, 0.071 and 0.051 mM⁻¹ for Fru6P, Fru(1,6) P_2 , NADP⁺ and NAD⁺ respectively.

Flux ratios, steady-state velocity measurements and 'pulse-chase' observations

All flux and steady-state velocity measurements were carried out at 30°C in 30 mM-triethanolamine/HCl buffer, pH 8, containing 50 mM-KCl, 6 mM-(NH₄)₂SO₄, 1 mM-dithiothreitol and sufficient MgCl₂ to give free Mg²⁺ concentration of 4 mM. Tetraethylammonium chloride was added to

maintain the ionic strength at 0.2. 'Pulse-chase' measurements were made with the same buffer and free Mg^{2+} in the 'pulse' solution at room temperature (20–22°C). The ionic strength was 0.08 in the 'pulse' solution, except in some measurements where tetraethylammonium chloride was added to raise the ionic strength to 0.2.

Ratio Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{Fru6P}$ / Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{ATP}$

This ratio was measured as a function of Fru6P concentration at zero ATP concentration. The experimental procedure involved carrying out the reaction in the direction of ATP synthesis in the presence of $[^{14}\text{C}]\text{Fru6P}$. The incorporation of label in $\text{Fru}(1,6)\text{P}_2$ and the amount of the reaction were measured. To facilitate trapping of label in $\text{Fru}(1,6)\text{P}_2$, a high concentration of $\text{Fru}(1,6)\text{P}_2$ was used. The ADP concentration was kept constant. The flux ratio increases from unity as the Fru6P concentration is increased and reaches a plateau of about 3.2 at Fru6P concentrations above 0.3 mM (Fig. 1a). In the measurements of the flux ratio by the technique described it is important that the ATP should be maintained at a sufficiently low concentration for the enzymic reaction between ATP and Fru6P to occur at a negligible rate, since spuriously high values will be obtained for the flux

ratio if this reaction should occur. The experiments at the higher Fru6P concentrations were therefore repeated with $[^{32}\text{P}]\text{ATP}$ rather than $[^{14}\text{C}]\text{Fru6P}$ since the formation of $[^{32}\text{P}]\text{Fru}(1,6)\text{P}_2$ will indicate the occurrence of this reaction. A small incorporation of label of ^{32}P into $\text{Fru}(1,6)\text{P}_2$ was observed at 0.5 mM- Fru6P , but it affected the ratio by less than 5%. The effect was smaller at lower concentrations of Fru6P and was negligible below 0.2 mM. The data in Fig. 1(a) have been appropriately corrected. For details of the calculation see Appendix II.

Ratio Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{ATP}$ / Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{Fru6P}$

This ratio was measured as a function of ATP concentration at zero Fru6P concentration. The enzymic reaction was carried out in the direction of ATP synthesis, and the incorporation of label into $\text{Fru}(1,6)\text{P}_2$ derived from $[^{32}\text{P}]\text{ATP}$ and the extent of the reaction were determined. A high concentration of $\text{Fru}(1,6)\text{P}_2$ was used to trap the label in the diphosphate, and the ADP concentration was kept constant. The ratio increases from unity to reach a plateau of about 1.4 at concentrations of ADP greater than 0.8 mM (Fig. 1b). For reasons discussed immediately above, it was important that the Fru6P concentration be maintained at a sufficiently low value for the enzymic reactions

Fig. 1. Flux ratios related to the addition of substrates in the reaction catalysed by phosphofructokinase (a) The ratio Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{Fru6P}$ / Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{ATP}$ was measured as a function of Fru6P concentration by determining the incorporation of label from $[^{14}\text{C}]\text{Fru6P}$ into $\text{Fru}(1,6)\text{P}_2$ with the reaction proceeding in the direction of ATP synthesis. The ATP concentration was maintained at zero. For details of the experimental procedure and calculations, see the Materials and methods section and the Results and discussion section. The continuous line is a hyperbola drawn as described under 'Interpretation of results' in the Results and discussion section. (b) The ratio Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{ATP}$ / Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{Fru6P}$ was measured as a function of ATP concentration by determining the incorporation of label from $[^{32}\text{P}]\text{Fru}(1,6)\text{P}_2$ into ATP while the reaction was proceeding in the direction of ATP synthesis. The concentration of Fru6P was maintained at zero with phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. For details of experimental procedures and calculations see the Materials and methods section and the Results and discussion section. The continuous line is a hyperbola drawn as described under 'Interpretation of results' in the Results and discussion section.

between Fru6P and ATP to occur at a negligible rate. Experiments were therefore carried out with [^{14}C]Fru6P rather than [^{32}P]ATP. At 1.25 mM-ATP, the incorporation of label into Fru(1,6) P_2 indicated that there was sufficient reaction to increase the apparent ratio by 15%, but at 1 mM-ATP the factor was 6% and at 0.5 mM and below the factor was less than 5%. The data in Fig. 1(b) have been corrected by these factors.

Ratio Flux of ATP→Fru(1,6) P_2 /Flux of ATP→ADP

This ratio was measured at two Fru(1,6) P_2 concentrations and at zero ADP concentration (Table 1). To determine the ratio, the enzyme reaction was carried out in the direction of Fru(1,6) P_2 synthesis in the presence of [^{32}P]Fru(1,6) P_2 , and incorporation of label in ATP and the amount of reaction were measured. A high concentration of ATP was used to trap any label. However, no significant labelling of ATP was observed even at low Fru(1,6) P_2 and Fru6P concentrations, when incorporation of label into ATP is theoretically most likely to take place. The flux ratios therefore did not significantly differ from unity under any of the conditions. Table 1 also gives values for the calculated flux ratios assuming an ordered dissociation of products, with Fru(1,6)- P_2 first to leave the enzyme. In all cases these calculated values are considerably greater than the random scatter of the observations.

Ratio Flux of ATP→ADP/Flux of ATP→Fru(1,6) P_2

Measurements of this ratio were made as a function of ADP concentration at zero Fru(1,6) P_2 concentration. The reaction was carried out in the direction of Fru(1,6) P_2 synthesis in the presence of [^{14}C]ADP, and incorporation of label in ATP was measured. To trap the label in the ATP, a high concentration of ATP was used and a saturating concentration of Fru6P (5 mM) was also employed. A high concentration of Fru6P must decrease the transfer of label from ADP to ATP and correspondingly decrease the flux ratio towards unity, but was necessary since preliminary experiments had shown that the enzyme catalyses the exchange of label from [^{14}C]ADP to ATP at a rate of 0.1% of the maximum forward reaction in the absence of Fru6P. This side reaction, which appears to be related to the ATPase activity of the enzyme (Bloxxham & Lardy, 1973), would have increased the apparent value of the flux ratio. The results indicate that the flux ratio is not significantly greater than unity (Fig. 2). In contrast, the flux ratio should be substantially greater than unity if the dissociation of products should be ordered with ADP first to leave the enzyme (line 1, Fig. 2).

'Pulse-chase' experiments

[^{32}P]ATP trapping. In these experiments the enzyme was incubated with [^{32}P]ATP ('pulse' solution), and the mixture was rapidly added to a 'chase' solution containing Fru6P and a large

Table 1. Flux-ratio measurements to determine whether Fru(1,6) P_2 may dissociate from phosphofructokinase before ADP. The ratio Flux of ATP→Fru(1,6) P_2 /Flux of ATP→ADP was determined from the incorporation of label from [^{32}P]Fru(1,6) P_2 into ATP with the reaction proceeding in the direction of Fru(1,6) P_2 synthesis. The ADP concentration was maintained at zero. For experimental details see the Materials and methods section. The measured and theoretical values of the flux ratios were calculated as described in Appendix II with the use of the mean concentrations of Fru(1,6) P_2 and Fru6P over the experimental periods. Note that the method of measurement determines the difference in flux ratio from unity.

	[Fru6P] (mM)	[Fru(1,6) P_2] (mM)	Incorporation of label into ATP (% of total label)	Flux of ATP→Fru(1,6) P_2 / Flux ATP→ADP	
				Measured	Calculated for ordered dissociation
Initial	1.00	0.05	−0.3	0.998	1.14
Final	0.82	0.23			
Initial	1.00	0.05	−2.5	0.84	1.64
Final	0.90	0.60			
Initial	<0.08	0.05	+0.3	1.004	>1.10
Final	<0.08	0.12			
Initial	<0.06	0.05	−1.0	0.99	>1.23
Final	<0.06	0.19			
Initial	<0.13	0.50	+2.6	1.23	>1.79
Final	<0.13	0.56

Fig. 2. Flux-ratio measurements to determine whether ADP may leave phosphofructokinase before Fru(1,6)P₂ dissociates

The ratio Flux of ATP→ADP/Flux of ATP→Fru(1,6)P₂ was determined from the incorporation of label into ATP from [8-¹⁴C]ADP with the reaction proceeding in the direction of Fru(1,6)-P₂ synthesis. Samples were removed during the course of the reaction and analysed for Fru(1,6)P₂ and ADP as described in the Materials and methods section. Line 1 is the theoretical line calculated from the Dalziel coefficients (see Appendix II) for an ordered dissociation of products with Fru(1,6)P₂ the last to leave the enzyme. For experimental details see the Materials and methods section.

excess of unlabelled ATP. Excess labelling was observed in the Fru6P formed in the 'chase' solution, indicating that in the 'pulse' solution an enzymically active E·ATP complex is formed that is converted into products in the 'chase' solution before the bound ATP exchanges with ATP in solution. In one group of experiments the excess label was measured as a function of ATP concentration in the 'pulse' solution. A Scatchard plot showed hyperbolic binding of ATP with a K_D of 46 μ M and a maximum ATP binding of 1.1 mol/mol of enzyme subunit (Fig. 3a). In a second group of experiments the ATP concentration was maintained constant in the 'pulse' solution and the Fru6P concentration was varied in the 'chase' solution. A reciprocal plot showed hyperbolic kinetics and indicated that the data at different ATP concentration were obtained under essentially saturating conditions with respect to Fru6P concentration (Fig. 3b).

The ionic strength in the 'pulse' solution in the experiments described above was 0.08. To investigate the effects of ionic strength on the K_D for ATP, parallel experiments were carried out with 21 μ M-ATP in the 'pulse' solution. In one experiment the ionic strength was 0.08; in the other ionic strength was raised to 0.2 with tetraethylammonium chloride. Duplicate observations indicated that 16.5% label was trapped in Fru(1,6)P₂ at the lower ionic strength and 11.3% at the higher

Fig. 3. [γ -³²P]ATP trapping experiments to demonstrate the formation of an enzyme-ATP complex. At room temperature (20–22°C) 10 μ l of [γ -³²P]ATP (0.43 mM, except where indicated) was added to 200 μ l of a 'pulse' solution containing 0.33 mg of phosphofructokinase, 26 μ M-ATP (associated with the enzyme), 50 mM-KCl, 6 mM-(NH₄)₂SO₄, 1 mM-dithiothreitol, sufficient MgCl₂ to give a free Mg²⁺ concentration of 4 mM and 30 mM-triethanolamine/HCl buffer, pH 8. After approx. 2 s, 100 μ l of this solution was added to 900 μ l of 'chase' solution containing 100 mM-ATP, 10 mM-Fru6P (except where indicated), 50 mM-KCl, 6 mM-(NH₄)₂SO₄, 1 mM-dithiothreitol, sufficient MgCl₂ to give a free Mg²⁺ concentration of 4 mM and 30 mM-triethanolamine/HCl buffer, pH 8. After approx. 2 s, 1 ml of 1 M-HClO₄ was added. The amount of label in Fru(1,6)P₂ was determined. For further details see the Materials and methods section. (a) Scatchard plot obtained by variation of the concentration of ATP in the 'pulse' solution. (b) Variation of the concentration of Fru6P in the 'chase' solution.

value. The conditions in the 'chase' solution were virtually unchanged by the addition of tetraethylammonium chloride. Thus the fraction of label bound to the enzyme that is converted into products should be the same in both cases. With this assumption it follows that raising the ionic strength from 0.08 to 0.2 increases the K_D from 46 μM to 92 μM .

[$U\text{-}^{14}\text{C}$]Fru6P trapping. [^{14}C]Fru6P experiments were carried out at three concentrations of phosphofructokinase, with the use of enzyme from which ATP had been removed by two methods. At concentrations of 1.67 mg/ml (two experiments) and 10 mg/ml (one experiment) ATP was removed by treating the enzyme with unlabelled Fru6P: at a concentration of 6.4 mg/ml (two experiments) ATP was removed by charcoal treatment of the enzyme solution. In all cases the radioactivity transferred to Fru(1,6) P_2 was less than 5% of that expected if an E·Fru6P complex were formed that did not exchange with the solution.

Steady-state velocity measurements

Reciprocal plots of the forward velocity as a function of ATP and Fru6P concentrations were substantially linear (Fig. 4), and least-squares fitting gave the following Dalziel coefficients: $\phi_0 = 4.62 \times 10^{-3} \text{ s}$, $\phi_{\text{Fru6P}} = 4.2 \times 10^{-1} \mu\text{M}\cdot\text{s}$, $\phi_{\text{ATP}} = 6.94 \times 10^{-1} \mu\text{M}\cdot\text{s}$ and $\phi_{\text{Fru6P,ATP}} = 30.5 \mu\text{M}^2\cdot\text{s}$. (ϕ_{Fru6P} , ϕ_{ATP} and $\phi_{\text{Fru6P,ATP}}$ refer

Fig. 4. Steady-state kinetics of phosphofructokinase in the direction of Fru(1,6) P_2 synthesis

Reciprocal plots of initial velocity against concentration of ATP are shown. The concentrations of Fru6P were as follows: $\circ$, 30 μM ; $\triangle$, 60 μM ; $\square$, 100 μM ; ∇ , 300 μM ; $\bullet$, 3 mM. The reaction was carried out at 30°C in a volume of 3 ml containing 30 mM-triethanolamine/HCl buffer, pH 8, 50 mM-KCl, 6 mM-(NH_4) $_2\text{SO}_4$, 1 mM-dithiothreitol and sufficient tetraethylammonium chloride to give an ionic strength of 0.2. For further details see the Materials and methods section.

to the ϕ terms associated with Fru6P, ATP and Fru6P and ATP in the denominator in the expression for the reciprocal velocity; see Appendix II). However, the flux-ratio data clearly show that the addition of substrates is neither ordered, nor rapid and random, despite the apparent linearity of the data. For a random addition of substrate (Scheme 1), with the use of the flux-ratio and 'pulse-chase' data it is necessary only to assume values for k_{+3} and k_{+1} to obtain the theoretical velocity-versus-substrate curves. The lines in Fig. 4, which are substantially linear, were constructed by choosing appropriate values for these rate constants.

Measurements of the reverse velocity as a function of Fru(1,6) P_2 and ADP concentrations gave reciprocal plots that were approximately linear (Fig. 5). Visual fitting of the data gave the following Dalziel coefficients: ϕ_0 0.2 s, $\phi_{\text{Fru(1,6)P}_2}$ 804 $\mu\text{M}\cdot\text{s}$, ϕ_{ADP} 541 $\mu\text{M}\cdot\text{s}$ and $\phi_{\text{Fru(1,6)P}_2, \text{ADP}}$ $1.68 \times 10^5 \mu\text{M}^2\cdot\text{s}$.

Interpretation of results

Mg^{2+} binding by the substrates. In the present work no attempt was made to establish whether the free substrates or the substrates bound to Mg^{2+} were the true substrates for the enzyme. Since the free [Mg^{2+}] was kept constant (4 mM), the proportions of free and liganded forms were constant and all calculations are based on the total concentrations of the substrates.

Addition of substrates. A ratio Flux of Fru(1,6)- $P_2 \rightarrow$ Fru6P/Flux of Fru(1,6) $P_2 \rightarrow$ ATP greater than

Fig. 5. Steady-state kinetics of phosphofructokinase in the direction of ATP synthesis

Reciprocal plots of initial velocity against concentration of Fru(1,6) P_2 are shown. The concentrations of ADP were as follows: ∇ , 30 μM ; $\circ$, 45 μM ; $\bullet$, 60 μM ; $\triangle$, 100 μM ; $\blacktriangle$, 300 μM . The reaction conditions were as indicated in Fig. 4 legend. The reaction was monitored by the addition of (final concentrations) phosphoglucose isomerase (10 $\mu\text{g}/\text{ml}$), glucose-6-phosphate dehydrogenase (10 $\mu\text{g}/\text{ml}$) and NADP $^+$ (0.33 mM). Phosphofructokinase (17 $\mu\text{g}/\text{ml}$) was added to start the reaction.

unity (Fig. 1a) is unequivocal evidence that one pathway for the reaction involves the addition of ATP first to the enzyme in a rate-limiting step. Isotope effects could cause errors in the determination of the fluxes, but with ^{14}C and ^{32}P such effects will be very small. The ratio rises hyperbolically to a plateau rather than linearly as the Fru6P concentration is increased, and therefore the substrates can also add in the reverse order. For such random addition of substrates the flux ratio in the absence of ATP should obey the following expression (Britton & Dann, 1978):

$$\frac{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{Fru6P}}{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{ATP}} = \frac{1 + \alpha[\text{Fru6P}]}{1 + \beta[\text{Fru6P}]}$$

A fit to the experimental points is obtained with $\alpha = 22\text{ mM}^{-1}$ and $\beta = 6\text{ mM}^{-1}$ (line 1, Fig. 1a), which gives a plateau value (R_A) for the hyperbola of 3.67.

Similar considerations apply to the ratio Flux of Fru(1,6)P₂ → ATP / Flux of Fru(1,6)P₂ → Fru6P. The ratio rises above unity, and therefore one pathway for the reaction must involve the addition of Fru6P to the free enzyme in a rate-limiting step. Further, the ratio rises hyperbolically to a plateau, again indicating that the substrate can add in the reverse

order. In the absence of Fru6P the flux ratio should obey the equation (Britton & Dann, 1978):

$$\frac{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{ATP}}{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{Fru6P}} = \frac{1 + \gamma[\text{ATP}]}{1 + \delta[\text{ATP}]}$$

To fit the experimental points the values $\gamma = 9\text{ mM}^{-1}$ and $\delta = 6.4\text{ mM}^{-1}$ were assigned, giving a plateau value (R_B) of the hyperbola of 1.41.

The plateau values R_A and R_B [Figs. 1(a) and 1(b); see also above] must be related by the expression (Britton & Dann, 1978):

$$R_B < R_A / (R_A - 1)$$

This relationship makes the conventional assumption that the rate constants involved in the addition of the substrates are independent of the concentration of these substrates. From the relationship the experimental value of R_A (3.67) gives a maximum value for R_B (1.37) that is virtually identical with the experimentally determined value (1.41). It follows (Britton & Dann, 1978) that $k_{-2}, k_{-7} \ll k_{+3}$ (Scheme 1a). Thus when the ternary complex is formed it is very unlikely that either ATP or Fru6P will dissociate again: on most occasions release of products [Fru(1,6)P₂ and ADP] will occur. If this is the case, then $k_{-2}/k_{-7} = (R_A - 1) = 2.67$. The pro-

Scheme 1. (a) Rate constants for a sequential reaction involving a random binding of substrates and dissociation of products; and (b) rate constants calculated for rabbit muscle phosphofructokinase at pH 8, 10.2, 4 mM free Mg^{2+} and 30°C . A = ATP, B = Fru6P, P = ADP, and Q = Fru(1,6)P₂. The steps involving the dissociation of products are assumed to be rapid (see the text). Rate constants have been calculated per subunit assuming an M_r of phosphofructokinase of 380000 and four subunits (Paetkau & Lardy, 1967).

portion of the enzyme reaction following the two possible pathways of substrate addition depends on this ratio (Britton & Dann, 1978). Thus at low concentrations of substrates 72% of the reaction follows the pathway with ATP adding first to the free enzyme and 28% follows the alternative pathway.

'Pulse-chase' labelling of Fru(1,6) P_2 (isotope trapping) with [γ - ^{32}P]ATP (Uyeda, 1970) was demonstrated under rigorous conditions, and its dependence on ATP concentration in the 'pulse' solution and Fru6P concentration in the 'chase' solution was investigated. Isotope trapping indicates that the enzyme and ATP form an enzymically active binary complex that dissociates slowly relative to the rate of formation of products in the 'chase' solution (Rose *et al.*, 1974). It is therefore further strong evidence that one pathway of enzyme reaction involves ATP adding first to the free enzyme in a rate-limiting step. Extrapolation indicates a maximum of 4.5 mol of ATP bound/mol of enzyme. This value, which is in agreement with the conclusion of Paetkau & Lardy (1967) that the enzyme is a tetramer, additionally indicates that the rate of any dissociation of ATP from the ternary complex must be small compared with the rate of conversion of ternary complex into products [$k_{-7} \ll k_{+3}$; Scheme 1(a), assuming a rapid dissociation of products]. Further, if the formation of the E·ATP complex is to be represented by a two-stage process

in which the first step is rapid (Britton, 1977), the equilibrium for the isomerization step must lie far to the right. Whereas at 10.08 the dissociation constant for the complex is 46 μM , at 10.2 the dissociation constant is 92 μM . The larger value at the higher ionic strength may be attributed to changes in activity coefficients.

No isotope trapping could be demonstrated when [^{14}C]Fru6P was incubated with the enzyme in the 'pulse' solution, despite the evidence from the flux ratios that Fru6P binds to the enzyme to form an enzymically active binary complex. This result may indicate that the dissociation constant for the binding of Fru6P to enzyme is large and that very little Fru6P is bound to the enzyme under the experimental conditions used ($K_D > 0.5\text{ mM}$). Alternatively, Fru6P may dissociate rapidly from the ternary complex compared with the rate of conversion into products [$k_{-2} \gg k_{+3}$; Scheme 1(a), assuming a rapid dissociation of products] or Fru6P may dissociate rapidly from the binary complex [$k_{-6} \gg k_{+7}[\text{ATP}]/(k_{-7} + k_{+3})$; Scheme 1(a), assuming a rapid dissociation of products]. Of these two latter possibilities, the first is incompatible with the flux-ratio data that have already been

discussed, and the second is inconsistent with the calculated values for the rate constants (see below). It must be concluded that the failure to observe 'pulse-chase' labelling with Fru6P is therefore due to a large K_D of the enzyme for Fru6P. This conclusion is in contrast with the observations of relatively tight binding of Fru6P by the enzyme (Hill & Hammes, 1975; Kemp & Krebs, 1967; Lorensen & Mansour, 1969; Jones *et al.*, 1975). It would seem that the complexes that have been reported are not productive complexes or that the Fru6P is bound at the allosteric site. Most of the observations have also been made in the absence of Mg^{2+} , when the enzyme shows only little activity.

When the flux-ratio approach was used to examine the dissociation of products, the ratios (Flux of ATP→ADP/Flux of ATP→Fru6P and its reciprocal) were found to be not significantly different from unity. As described in Appendix II the expected values for these flux ratios for ordered dissociations of the products may be calculated from the Dalziel coefficients for the reverse reaction and the observed values of the ratio Flux of Fru(1,6) P_2 →Fru6P/Flux of Fru(1,6) P_2 →ATP. These calculations show that the flux ratios for either order of dissociation should have been considerably greater than unity. Ordered dissociations with Fru(1,6) P_2 or ADP dissociating first from the ternary complex are therefore excluded. This conclusion depends on the assumption that the enzyme is not under allosteric control by the substrates or products at pH 8, since such interactions could affect the steady-state velocity curves. A flux ratio greater than unity depends on a dissociation of product from the appropriate binary complex that is slow, and correspondingly the present flux-ratio data might suggest that the dissociations of the products from their binary complexes must be rapid. However, when the dissociation of products is random, the flux ratios may approximate to unity even when one or both of these steps are slow, provided that the equilibrium lies far towards the right. Again, the hyperbolic kinetics of the reverse reaction suggest that the dissociations of the products from the binary complexes are rapid, but only in comparison with the velocity of the reverse reaction.

Phosphofructokinase catalyses the exchange of a phosphate group between ATP and ADP (Uyeda, 1970; Hulme & Tipton, 1971; Hanson *et al.*, 1973) and between Fru6P and Fru(1,6) P_2 (Lowry & Passonneau, 1966; Hulme & Tipton, 1971), suggesting that the enzyme has a ping-pong mechanism. However, the rates of exchange are very much less than the rate of the normal enzyme reaction. The present data also provide evidence to exclude the ping-pong mechanism as a major pathway. Thus for a ping-pong mechanism the ratio Flux

of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{ATP}/\text{Flux}$ of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{Fru6P}$ should have been unity. Further, the ratios Flux of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{Fru6P}/\text{Flux}$ of $\text{Fru}(1,6)\text{P}_2 \rightarrow \text{ATP}$ should have been very much greater than the observed values (see Appendix II).

From the data just discussed, the binding of substrates to the enzyme is random and there is a random dissociation of products. The values of k_{+1} , k_{-1} , k_{+2} , k_{+6} , k_{-6} and k_{+7} (Scheme 1a) are related by the flux-ratio and 'pulse-chase' data, and if a value for k_{+1} is assumed all of these constants are determined (see Appendix III). If the dissociation of products from the ternary complex and from their respective binary complexes was assumed to be rapid, the velocity in the forward direction [formation of $\text{Fru}(1,6)\text{P}_2$] is thus determined only by k_{+1} and k_{+3} (Scheme 1a). By appropriate adjustment of these constants (see Appendix III) the theoretical lines in Fig. 4 were obtained. From the kinetics of the reverse reaction, the remaining rate constants k_{-3} , k_{-2} and k_{-7} were found, and the data are shown in Scheme 1(b). Despite the fact that the data indicate that the mechanism is non-rapid random, the reciprocal plots of the steady-state velocities are essentially linear (Fig. 4). The values also show that the binding of either substrate markedly increases the affinity of the enzyme for the other substrate. If the dissociations of the products from the ternary complex to give the binary complexes are slow, the pattern of the steady-state kinetics will be unchanged, but k_{+3} becomes a composite constant involving the rate of dissociation of these products as well as the rate constant for the interconversion of the ternary complex. If the dissociations of the binary complexes of the products with the enzyme are slow (see previous discussion), k_{+3} will incorporate these constants as well. However, the values k_{+1} , k_{-1} , k_{+2} , k_{+6} , k_{-6} and k_{+7} will not be changed, and there should be only minor changes in k_{-2} and k_{-7} .

In a realistic enzyme mechanism the binding of a substrate to an enzyme to form a binary complex must be a multistage process. As discussed elsewhere (Britton, 1977), in the steady state such processes cannot be distinguished from a single rapid binding step followed by a slow isomerization of the complex. An isomerization of the $\text{E} \cdot \text{Fru6P}$ binary complex of this type improves the fit of the data to the experimental points at high Fru6P concentrations (Fig. 4), but it is doubtful if this can be regarded as sufficient evidence for the existence of such a step (see Appendix III). An isomerization of the $\text{E} \cdot \text{ATP}$ complex also need not substantially alter the kinetics. As a consequence of introducing these steps, the rate constants for the isomerization of the ternary complex will be increased and the rate constants shown are

therefore to be regarded as minimum values. However, if the isomerizations of the binary complexes are made sufficiently slow to increase greatly the rate constants for the isomerization of the ternary complex, the fit to the experimentally determined data deteriorates considerably.

A number of workers have reported binding of ATP by phosphofructokinase (see Uyeda, 1979). Using etheno-ATP, Roberts & Kellett (1980) reported that binding of the ATP to the active site of phosphofructokinase takes place by a 'prior isomerization pathway' in which there is a slow isomerization of the enzyme followed by a relatively rapid addition of the nucleotide. These observations are not compatible with the present data, since the dissociation of ATP from the binary complex must be slow to account for the flux-ratio data. However, the observations reported by Roberts & Kellett (1980) were made at a very different pH (6.8) and were based on fluorescence changes observed on binding of the fluorescent analogue of ATP. In this respect, rate constants determined from the kinetics of the enzyme reaction relate to when the $\text{E} \cdot \text{ATP}$ complex adopts a suitable configuration to accept a molecule of Fru6P . The observed fluorescence changes need not be associated with this event.

General discussion

The application of flux-ratio and 'pulse-chase' techniques has shown that there are two classes of phosphokinase enzymes, as far as mechanism is concerned. With the glucokinase enzymes, flux-ratio studies have indicated a highly ordered addition of substrates with the phosphate acceptor binding first [yeast hexokinase (Britton & Clarke, 1972); rat liver glucokinase (Gregoriou *et al.*, 1981); rat muscle glucokinase (Gregoriou *et al.*, 1983)]. 'Pulse-chase' studies with yeast hexokinase are also entirely consistent with and support such an addition (Rose *et al.*, 1974). With these enzymes, therefore, the side reaction of hydrolysis of ATP with the production of P_i may be prevented by the phosphate acceptor being in place on the enzyme before the phosphate donor is bound. In contrast, with pyruvate kinase flux-ratio and 'pulse-chase' studies have shown that the preferred pathway involves the phosphate donor, phosphoenolpyruvate, binding first to the enzyme in a step that is partially rate-limiting, although a substantial part of the reaction also takes place by the substrates binding in reverse order (Dann & Britton, 1978). The present work indicates a similar pattern with phosphofructokinase. In these enzymes, therefore, the phosphate group that is to be donated must not be activated until the phosphate acceptor is bound and the ternary complex is formed. Despite the random addition,

with both pyruvate kinase (Dann & Britton, 1978) and phosphofructokinase (present data) binding of one substrate markedly increases the affinity of the enzyme for the other substrate. Binding of both of the substrates therefore produces progressive changes in the enzyme structure leading to the formation of the ternary complex.

The present work demonstrates the value of the 'non-equilibrium' method of determining flux ratios of the type Flux of $Q \rightarrow B$ /Flux of $Q \rightarrow A$. It directly measures the difference in the flux ratio from unity, and this was of particular value in the present study since the ratio Flux of Fru(1,6)- $P_2 \rightarrow$ ATP/Flux of Fru(1,6)- $P_2 \rightarrow$ Fru6P was sufficiently close to unity to make measurement of the individual fluxes impracticable. The 'non-equilibrium' method also simplifies the experimental procedure, since only one radioactively labelled substrate is used at a time. However, the present work shows that when it is employed to study the addition of substrates the concentration of substrate at 'zero' concentration must be extremely small, to prevent reaction of the substrate with the enzyme. In practice it would seem important that this should be checked with suitably labelled substrate.

In the present study and in work on pyruvate kinase (Dann & Britton, 1978) 'pulse-chase' studies were made in addition to measurement of flux ratios. As discussed elsewhere (Britton, 1984), the information provided by these tests is similar to but not identical with that obtained from flux studies. Thus these tests can provide unequivocal evidence for the binding of a substrate to an enzyme in a rate-limiting step. The measurements can also provide a dissociation constant for the binding of the substrate, although the value obtained will strictly apply only to the first turnover. However, with both pyruvate kinase and phosphofructokinase, 'pulse-chase' measurements gave no indication that the substrates can add in either order. With pyruvate kinase, no 'pulse-chase' was observed with ADP (Dann & Britton, 1978), and no 'pulse-chase' was found in the present work with phosphofructokinase with Fru6P. However, flux-ratio measurements with both enzymes have clearly shown that Fru(1,6)- P_2 can add first to phosphofructokinase, and phosphoenolpyruvate can add first to pyruvate kinase, and that the addition of substrates is random with both enzymes.

The flux ratios concerned with the dissociation of products did not differ significantly from unity. The evidence for a random dissociation Fru6P and ADP therefore depends on the fact that the flux ratios for an ordered dissociation with either order of dissociation may be calculated from the steady-state kinetics and the fact that the values obtained

were much larger than those observed. Such evidence is less conclusive than if flux ratios greater than unity had been found. However, ratios of unity must be expected when the equilibrium constant for the reaction lies far to the right and there is a random dissociation. Even with ratios of unity, however, the technique would appear to offer the least equivocal method of determining how the substrates dissociate. In contrast, product-inhibition studies in the presence of a random dissociation of products will not in general yield linear reciprocal plots. The departure from linearity may not be noticeable over the range of concentrations used in any one experiment, but extrapolation of the line to the vertical axis under these conditions cannot be justified and may be misleading. To obtain more conclusive evidence about the order of dissociation of products it may be of value to develop the experimental technique so that very small departures of the flux ratio from unity may be detected. However, with phosphofructokinase, the contribution of the ATPase reaction has to be taken into account. With other enzymes there is the possibility that similar side reactions may occur, and the effect of these reactions on the flux ratios must be considered when small departures of the flux ratio from unity are observed.

We thank the Wellcome Trust for financial support.

References

- Bar-Tana, J. & Cleland, W. W. (1974) *J. Biol. Chem.* **249**, 1271-1276
- Belaich, J. P. & Sari, J. C. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 763-770
- Bloxham, D. P. & Lardy, H. A. (1973) *Enzymes 3rd Ed.* **8**, 239-278
- Britton, H. G. (1966) *Arch. Biochem. Biophys.* **117**, 167-183
- Britton, H. G. (1977) *Biochem. J.* **161**, 517-526
- Britton, H. G. (1984) in *Techniques in Protein and Enzyme Biochemistry* (Tipton, K. F., ed.), Elsevier, Limerick, in the press
- Britton, H. G. & Clarke, J. B. (1972) *Biochem. J.* **128**, 104p
- Britton, H. G. & Dann, L. G. (1978) *Biochem. J.* **169**, 29-37
- Burton, K. (1959) *Biochem. J.* **71**, 388-395
- Cleland, W. W. (1967) *Annu. Rev. Biochem.* **36**, 77-111
- Dann, L. G. & Britton, H. G. (1978) *Biochem. J.* **169**, 39-54
- Gregoriou, M., Trayer, I. P. & Cornish-Bowden, A. (1981) *Biochemistry* **20**, 499-506
- Gregoriou, M., Trayer, I. P. & Cornish-Bowden, A. (1983) *Eur. J. Biochem.* **134**, 283-288
- Hanson, R. L. (1970) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29** 408 (Abstr.)
- Hanson, R. L., Rudolph, F. B. & Lardy, H. A. (1973) *J. Biol. Chem.* **248**, 7852-7859

- Hill, D. E. & Hammes, G. G. (1975) *Biochemistry* **14**, 203–213
- Hulme, E. C. & Tipton, K. F. (1971) *Biochem. J.* **122**, 181–187
- Jones, R., Dwek, R. A. & Walker, I. O. (1975) *Eur. J. Biochem.* **60**, 187–198
- Kee, A. & Griffin, C. C. (1972) *Arch. Biochem. Biophys.* **149**, 361–368
- Kemp, R. G. & Krebs, E. (1967) *Biochemistry* **6**, 423–434
- Lorenson, M. Y. & Mansour, T. E. (1969) *J. Biol. Chem.* **244**, 6420–6431
- Lowry, O. H. & Passonneau, J. V. (1966) *J. Biol. Chem.* **241**, 2268–2279
- Martensen, T. M. & Mansour, T. E. (1976) *J. Biol. Chem.* **251**, 3664–3670
- Merry, S. & Britton, H. G. (1981) *Biochem. Soc. Trans.* **9**, 159P
- Paetkau, V. & Lardy, H. A. (1967) *J. Biol. Chem.* **242**, 2035–2042
- Parmeggiani, A., Luft, J. H., Love, D. S. & Krebs, E. G. (1966) *J. Biol. Chem.* **241**, 4625–4637
- Ray, W. J. & Roscelli, G. A. (1966) *J. Biol. Chem.* **241**, 2596–2602
- Roberts, D. & Kellett, G. L. (1980) *Biochem. J.* **189**, 561–567
- Rose, I. A., O'Connell, E. L. & Litwin, S. (1974) *J. Biol. Chem.* **249**, 5163–5168
- Rubio, V., Britton, H. G. & Grisolia, S. (1979) *Eur. J. Biochem.* **93**, 245–256
- Uyeda, K. (1970) *J. Biol. Chem.* **245**, 2268–2275
- Uyeda, K. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **48**, 193–244
- Wold, F. & Ballou, C. E. (1957) *J. Biol. Chem.* **227**, 301–312

APPENDIX I

Effects of the reverse reaction on flux ratios measured by the non-equilibrium method

To determine flux ratios by the non-equilibrium method, the enzyme reaction is carried out in the presence of a radioactive product, P, and the concentration of the other product, Q, is kept low enough, usually by a secondary enzyme reaction, to prevent any substantial back reaction. If A and B are the substrates and R is the product of the secondary enzyme reaction, the steps may be represented as follows:

Considering A and Q it is possible to write:

$$\text{Net flux of } A \rightarrow Q = \text{Flux of } A \rightarrow Q - \text{Flux of } Q \rightarrow A$$

or

$$\text{Flux of } A \rightarrow Q = \text{Net flux of } A \rightarrow Q + \text{Flux of } Q \rightarrow A$$

Similarly:

$$\text{Flux of } A \rightarrow P = \text{Net flux of } A \rightarrow P + \text{Flux of } P \rightarrow A$$

However:

$$\begin{aligned} \text{Net flux of } A \rightarrow Q &= \text{Net flux of } A \rightarrow P = \\ &\text{Rate of chemical reaction} = v \end{aligned}$$

and, provided that there is no substantial conversion of A into products:

$$\begin{aligned} \text{Flux of } P \rightarrow A &= \text{net rate of conversion of labelled} \\ &\text{P into A} = p \end{aligned}$$

Thus:

$$\frac{\text{Flux of } A \rightarrow P}{\text{Flux of } A \rightarrow Q} = (v + p)/(v + \text{Flux of } Q \rightarrow A) \quad (1)$$

$$\text{or if } (\text{Flux of } Q \rightarrow A)/v = k$$

$$\frac{\text{Flux of } A \rightarrow P}{\text{Flux of } A \rightarrow Q} = \left(1 + \frac{p}{v}\right)/(1 + k) \quad (2)$$

$(1 + k)$ therefore represents the correction factor that must be introduced if there is a significant Flux of $Q \rightarrow A$. k may be determined by introducing labelled Q in trace quantity into the reaction mixture when a steady state has been reached. Then, if radioactivity n_A accumulates in A and n_R radioactivity appears in R:

$$k = n_A/n_R$$

APPENDIX II

Calculation of expected flux ratios for an ordered dissociation of products and for a ping-pong mechanism

For an ordered dissociation of products the flux ratios Flux of ATP→ADP/Flux of ATP→Fru(1,6)-P₂ and Flux of ATP→Fru(1,6)P₂/Flux of ATP→ADP may be predicted from the values for the reverse reaction by using the following procedure. Consider Scheme 1(a). From Britton & Dann (1978) for an ordered association of substrates with A adding before B, and random dissociation of products:

$$\frac{\text{Flux of Q} \rightarrow \text{B}}{\text{Flux of Q} \rightarrow \text{A}} = 1 + \frac{(k_{+4} + k_{+8})k_{+2}k_{+3}[\text{B}]}{k_{-1}[k_{+3}(k_{+4} + k_{+8}) + k_{-2}(k_{+4} + k_{+8})(1 + Y[\text{P}])]} \quad (1)$$

where

$$Y = \frac{k_{-2}k_{-4}k_{-3}k_{+4}}{k_{+5}(k_{+4} + k_{+8})[(k_{+3} + k_{-2})(k_{+4} + k_{+8}) + k_{-2}k_{-3}] \left(1 + \frac{k_{+8}k_{-4}[\text{P}]}{k_{+5}(k_{+4} + k_{+8})}\right)}$$

From eqn. (1) it follows that:

$$\frac{\text{Flux Q} \rightarrow \text{B}}{\text{Flux Q} \rightarrow \text{A}} = 1 + \frac{\phi_{\text{A}}[\text{B}]}{\phi_{\text{AB}}(1 + Y[\text{P}])} \quad (2)$$

and

$$\frac{\text{Flux of A} \rightarrow \text{P}}{\text{Flux of A} \rightarrow \text{Q}} = 1 + \frac{Y[\text{P}]}{[1 + (\phi_{\text{A}}/\phi_{\text{AB}})][\text{B}]} \quad (3)$$

and

$$\frac{\text{Flux of B} \rightarrow \text{P}}{\text{Flux of B} \rightarrow \text{Q}} = 1 + Y[\text{P}] \quad (4)$$

Eqns. (1) and (4) correspond to eqns. (21) to (25) of Britton & Dann (1978), and are reproduced here since the original equations contained errors. In Britton & Dann (1978) Y was determined from eqn. (3) or (4) as appropriate, and the theoretical flux ratio for an ordered association of the substrates with the enzyme was derived from eqn. (2).

Since Y contains a term in $[\text{P}]$, all observations were made at the same concentration of P . The same procedure may be used to determine the theoretical flux ratios for an ordered dissociation of products by considering the reaction in the reverse direction. Thus, if the dissociation of the products is ordered with ADP dissociating first from the ternary complex:

$$\frac{\text{Flux of ATP} \rightarrow \text{ADP}}{\text{Flux of ATP} \rightarrow \text{Fru(1,6)P}_2} = 1 + \frac{\phi_{\text{Fru(1,6)P}_2}[\text{ADP}]}{\phi_{\text{Fru(1,6)P}_2, \text{ADP}}(1 + Y'[\text{Fru6P}])} \quad (5)$$

And if Fru(1,6)P₂ dissociates first from the ternary complex:

$$\frac{\text{Flux of ATP} \rightarrow \text{Fru(1,6)P}_2}{\text{Flux of ATP} \rightarrow \text{ADP}} = 1 + \frac{\phi_{\text{ADP}}[\text{Fru(1,6)P}_2]}{\phi_{\text{Fru(1,6)P}_2, \text{ADP}}(1 + Y''[\text{Fru6P}])} \quad (6)$$

Y' and Y'' may be determined from the relationships:

$$\frac{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{Fru6P}}{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{ATP}} = 1 + \frac{Y'[\text{Fru6P}]}{1 + \frac{\phi_{\text{Fru(1,6)P}_2}[\text{ADP}]}{\phi_{\text{Fru(1,6)P}_2, \text{ADP}}}} \quad (7)$$

and

$$\frac{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{Fru6P}}{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{ATP}} = 1 + Y''[\text{Fru6P}] \quad (8)$$

Since Y' and Y'' both contain terms in $[\text{Fru6P}]$, they should be determined at the Fru6P concentration used to measure the flux ratios given in eqns. (5) and (6).

For a ping-pong mechanism in which a phosphorylated enzyme is formed it may be shown that (Britton & Dann, 1978):

$$\frac{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{Fru6P}}{\text{Flux of Fru(1,6)P}_2 \rightarrow \text{ATP}} = 1 + \frac{\phi_{\text{Fru6P}}^{\text{Fru(1,6)P}_2} [\text{Fru6P}]}{\phi_{\text{Fru(1,6)P}_2}} + \frac{\phi_{\text{Fru(1,6)P}_2, \text{ADP}}^{\text{Fru6P}} [\text{Fru6P}]}{\phi_{\text{Fru(1,6)P}_2} [\text{ADP}]}$$

$$= 1 + \frac{\phi_{\text{Fru(1,6)P}_2}^{\text{Fru6P}} [\text{Fru6P}]}{\phi_{\text{Fru(1,6)P}_2}} + \frac{\phi_{\text{ADP}} [\text{Fru6P}]}{\phi_{\text{Fru6P}} [\text{ADP}]} \quad (9)$$

where $\phi_{\text{Fru(1,6)P}_2}$ etc. are Dalziel coefficients.

$\phi_{\text{Fru6P}} = 4.2 \times 10^{-1} \mu\text{M}\cdot\text{s}$ and $\phi_{\text{ADP}} = 541 \mu\text{M}\cdot\text{s}$.
The last term in eqn. (9) is therefore

$1288[\text{Fru6P}]/[\text{ADP}]$. It follows that the observed flux ratios are very much less than those predicted for a ping-pong mechanism.

Reference

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

APPENDIX III

Calculation of rate constants

Consider Scheme 1(a) of the main paper. Let $A = \text{ATP}$ and $B = \text{Fru6P}$ from Britton & Dann (1978).

$$k_{+2}/k_{-1} = \alpha \quad (1)$$

$$k_{+3}/k_{-6} = \gamma \quad (2)$$

and

$$(k_{-2} + k_{-7})/k_{-7} = R_A \quad (3)$$

Further:

$$k_{-1}/k_{+1} = K_A \quad (4)$$

and

$$k_{-6}/k_{+6} = K_B \quad (5)$$

where K_A and K_B are the dissociation constants for the EA and EB complexes respectively.

The rate constants for addition of substrates must also be related by the expression:

$$k_{+1}k_{+2}k_{-7}k_{-6} = k_{+6}k_{+7}k_{-2}k_{-1} \quad (6)$$

From eqns. (1)–(6):

$$k_{-1} = k_{+1}K_A \quad (7)$$

$$k_{+2} = k_{+1}\alpha K_A \quad (8)$$

$$k_{+7} = k_{+1}\alpha K_B/(R_A - 1) \quad (9)$$

$$k_{-6} = k_{+1}\alpha K_B/\gamma(R_A - 1) \quad (10)$$

$$k_{+6} = k_{+1}\alpha/\gamma(R_A - 1) \quad (11)$$

Also:

$$k_{-2}/k_{-7} = R_A - 1 \quad (12)$$

From Britton (1977):

$$(\text{EA}) = k_{+1}[\text{A}](\text{E})(k_{-1} + k_{+2}[\text{B}])^{-1} + k_{-2}(\text{EAB})(k_{-1} + k_{+2}[\text{B}])^{-1} \quad (13)$$

and

$$(\text{EB}) = k_{-6}[\text{B}](\text{E})(k_{-6} + k_{+7}[\text{A}])^{-1} + k_{-7}(\text{EAB})(k_{-6} + k_{+7}[\text{A}])^{-1} \quad (14)$$

Since k_{-2} , $k_{-7} \ll k_{+3}$, to a first approximation:

$$(\text{EA}) + (\text{EB}) = k_{+1}[\text{A}](\text{E})(k_{-1} + k_{+2}[\text{B}])^{-1} + k_{+6}[\text{B}](\text{E})(k_{-6} + k_{+7}[\text{A}])^{-1} \quad (15)$$

Further, the flux of E to EAB is equal to the flux of EAB to E under steady-state conditions (Britton, 1977). Thus, in the absence of products:

$$(\text{E})\{k_{+1}k_{+2}[\text{A}][\text{B}](k_{-1} + k_{+2}[\text{B}])^{-1} + k_{+6}k_{+7}[\text{A}][\text{B}](k_{-6} + k_{+7}[\text{A}])^{-1}\} = (\text{EAB})\{k_{-1}k_{-2}(k_{-1} + k_{+2}[\text{B}])^{-1} + k_{-6}k_{-7}(k_{-6} + k_{+7}[\text{A}])^{-1} + k_{+3}\} \quad (16)$$

If E_T = total amount of enzyme, $E_T = (\text{E}) + (\text{EA}) + (\text{EB}) + (\text{EAB})$. From this relationship and eqns. (9), (12), (15) and (17), the velocity of the enzyme reaction (v) in the absence of products is given by the expression:

$$v^{-1} = [k_{+3}(\text{EAB})]^{-1} = (Xk_{+1}^{-1} + k_{+3}^{-1}) E_T^{-1} \quad (17)$$

where, since $k_{-2}, k_{-7} \ll k_{+3}$:

$$X = \frac{1 + [A]\{K_A(1 + \alpha[B])\}^{-1} + [B]\{K_B(1 - \gamma[A])\}^{-1}}{\alpha[A][B]\{(1 + \alpha[B])\}^{-1} + \{(R_A - 1)(1 + \gamma[A])\}^{-1}} \quad (18)$$

K_B was assumed to be 1 mM (see below), K_A was obtained from the 'pulse-chase' data, and, with the values of α , γ and R_A , X was calculated for appropriate values of $[A]$ and $[B]$ (Fig. 4, main paper). Values of k_{+1} and k_{+3} were then chosen to fit the calculated data to the experimental points. From the k_{+1} value, k_{-1} , k_{+2} , k_{+6} and k_{+7} were derived from eqns. (7)–(12).

k_{+2} , k_{+6} and k_{+7} were derived from eqns. (7)–(12).

k_{-2} and k_{-7} were derived as follows:

$$k_{+6}k_{+7}k_{+3}K_{PQ} = k_{-6}k_{-7}k_{-3}K \quad (19)$$

where K_{PQ} is the appropriate Michaelis constant for the reverse reaction and K is the equilibrium constant for the overall reaction. From this equation the numerical value of the product $k_{-3}k_{-7}$ can be calculated. $K = 1387 \pm 359$ (mean $\pm$ S.E.M., $n = 4$) determined under the conditions of the flux-ratio experiments by the method of Hanson *et al.* (1973).

The maximum reverse velocity $v_{\max}^R$ is given by the expression:

$$\frac{1}{v_{\max}^R} = \frac{1}{E_T} \left[\frac{1}{k_{-3}} + \frac{1}{(k_{-2} + k_{-7})} \left(1 + \frac{k_{+3}}{k_{-3}} + \frac{k_{-2}}{k_{-1}} + \frac{k_{-7}}{k_{-6}} \right) \right] \quad (20)$$

From eqns. (20) and (3) the following quadratic equation in k_{-7} can be derived:

$$\frac{k_{-7}^2}{(k_{-3}k_{-7})} - \left(\frac{E_T}{v_{\max}^R} - \frac{k_{+3}}{R_A(k_{-3}k_{-7})} + \frac{R_A - 1}{k_{-1}} + \frac{1}{k_{-6}} \right) k_{-7} + \frac{1}{R_A} = 0$$

From the value of k_{-7} , k_{-2} and k_{-3} can be obtained from eqns. (3) and (19). These values inserted into eqn. (17) confirm that the second terms of the right-hand sides of eqns. (13) and (14) are small. In the final calculation these terms were introduced and the procedure was iterated.

In the above a value of K_B of 1 mM was assumed, representing the minimum compatible with the

absence of a 'pulse-chase' with Fru6P. A larger value for K_B yields a poorer fit to the experimentally determined data and gives a greater value for k_{+5} . If K_B is 10 mM, $k_{+5} = 21000 \text{ mM}^{-1}$. This appears large for a bi-molecular reaction involving ATP, and it may be concluded that K_B lies within the range 1–10 mM.

The fit to the experimental data is relatively close for values of $[B]$ from 0.03 to 0.3 mM, but at $[B]$ 3 mM the predicted velocities are higher than the experimental. A closer fit at high values of $[B]$ with a negligible effect on the calculated rates at low values of $[B]$ may be obtained if a second EB complex different from the complex shown in Scheme 1 (main paper) is also formed. This leads to an additional $[B]/K_B$ term, where K_B is the dissociation constant for the complex, in the numerator of the expression for X (eqn. 18). This complex may be a dead-end complex or an intermediate stage in the formation of the normal EB complex.

The numerical values of the constants k_{+1} , k_{-1} , k_{+2} , k_{+6} , k_{-6} and k_{+7} are relatively insensitive to the accuracy of the curve-fitting procedure. k_{+3} , however, is affected, since the X term (eqn. 17) is large. Thus k_{+3} and in consequence k_{-3} can only be regarded as approximate. However, k_{+3} cannot be much smaller than the calculated value (Scheme 1, main paper), since $k_{+3} \gg k_{-2}$, k_{-7} ; similarly, k_{-2}

and k_{-7} cannot be much larger than the calculated values. If k_{+3} is larger than calculated, k_{-3} will be

correspondingly increased and k_{-2} and k_{-7} will fall to minimum values of 3.9 and 1.5 s^{-1} respectively.

References

- Britton, H. G. (1977) *Biochem. J.* **161**, 517–526
- Britton, H. G. & Dann, L. G. (1978) *Biochem. J.* **169**, 29–37
- Hanson, R. L., Rudolph, S. B. & Lardy, H. A. (1973) *J. Biol. Chem.* **248**, 7852–7859