The Isotope-Exchange Reactions of Ox Heart Phosphofructokinase

By E. C. HULME AND K. F. TIPTON

*University of Cambridge, Department of Biochemistry, Tennis Court Road,
Cambridge CB2 1QW, U.K.*

(Received 16 October 1970)

1. Ox heart phosphofructokinase catalyses isotope-exchange reactions at pH 6.7 between ADP and ATP, and between fructose 6-phosphate and fructose 1,6-diphosphate, the latter reaction being absolutely dependent on the presence of the magnesium complex of ADP. 2. The reaction kinetics are hyperbolic with respect to substrate concentration for both exchange reactions (within the experimental error). 3. The influence of pH, AMP and citrate suggests that the fructose 6-phosphate–fructose 1,6-diphosphate exchange is subject to effector control, and is abolished by dissociation of the enzyme. 4. These results are discussed in relation to the reaction mechanism of the enzyme.

A great deal of kinetic work has been carried out on phosphofructokinases from various mammalian sources. One of the salient features to emerge from this work is that for a number of phosphofructokinases, plots of the reciprocal of the initial velocity against reciprocal concentration of one substrate at a series of fixed concentrations of the other substrate yield families of parallel lines at pH 8.0, both in the forward direction (Layzer, Rowland & Banks, 1969) and in the reverse direction (Lorenson & Mansour, 1968). This behaviour is seen with ox heart phosphofructokinase both at pH 8.0 and at 6.7 in the presence of saturating quantities of the positive effector AMP (E. C. Hulme, unpublished work). Kinetic behaviour of this type usually implies the dissociation of the first product from the enzyme before the combination of the second substrate, i.e. the reaction progresses via a free modified-enzyme intermediate (Cleland, 1963).

The work described was undertaken with the object of discovering whether or not the isotope-exchange reactions of ox heart phosphofructokinase are compatible with such a mechanism. The results presented here indicate that a free modified-enzyme intermediate plays no significant part in the mechanism of the overall reactions, which appears to be compulsory order with ATP combining first, and that the release of the first product (ADP) is contingent upon the binding of the second substrate (fructose 6-phosphate). The relative magnitudes of the velocity constants are responsible for the apparent nonconvergence of the double-reciprocal plots, by a depression of the binding constant for ATP. The reciprocal plots must in fact be weakly convergent.

Further, it appears that the binding of ATP and the transfer of its terminal phosphate group to the enzyme is not affected by the pH-dependent dissociation of the enzyme, and that this half-reaction is not subject to the effector control which is characteristic of phosphofructokinase.

In contrast with this, either the binding of fructose 6-phosphate, or the transfer to it of the phosphate group, appears to be a property of the associated form of the enzyme and strongly dependent upon the integrity of its subunit structure. This reaction appears to be the point at which effector control of the enzyme is exerted.

EXPERIMENTAL

Ox heart phosphofructokinase was prepared by the method of Frenkel (1966). This method gives specific activities of the order of 100 units/g of protein at 25°C. The enzyme was stored as a sludge in a 10 mM-potassium phosphate solution containing 60% (w/v) $(\text{NH}_4)_2\text{SO}_4$, pH 7.0.

Fructose 6-phosphate, fructose 1,6-diphosphate, AMP, ADP and ATP were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. $[^{14}\text{C}]\text{ADP}$ and $[^{14}\text{C}]\text{fructose 6-phosphate}$ were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals used were of AnalaR grade.

Exchange-reaction kinetics. Exchange studies were carried out at 25°C in a buffer containing 100 mM-KCl, 10 mM-MgSO₄ and 5 mM-dithiothreitol, and 10 mM-imidazole chloride for work at pH 6.7, or 10 mM-tris chloride for work at pH 8.0. Reactions were initiated by the addition of 6 μg of phosphofructokinase [freed from $(\text{NH}_4)_2\text{SO}_4$ by gel filtration on Sephadex G-25 (medium grade) (Pharmacia Fine Chemicals, Uppsala, Sweden), in a buffer containing 100 mM-KCl, 10 mM-MgSO₄ and

1 mM-imidazole chloride, pH 8.0] to 1 ml of the incubation mixture containing all the components required for the exchange, including the isotope at a specific radioactivity of $0.1 \mu\text{Ci/ml}$. After an appropriate length of time (5 or 10 min) the reaction was stopped by the addition of a sufficient quantity of 2M-HCl to adjust the pH to 2.0, followed by cooling in ice. Controls were carried out by omission of the enzyme.

The reaction mixture was then applied to a short column (6 cm $\times$ 1 cm) of DEAE-cellulose (H. Reeve Angel and Co. Ltd., London E.C.4, U.K.) equilibrated with a solution of 100 mM-KCl in 0.01 M-HCl, and eluted with the same solution. Fructose 6-phosphate is completely separated from fructose diphosphate, and ADP from ATP by using this system. The eluate was collected in two portions of about 15 ml each, containing the more- and less-strongly retarded component respectively. Samples (2 ml) were withdrawn from these and mixed with 14 ml of aqueous scintillator containing two parts of toluene to one part of Triton X-100, and 5 g of 2,5-diphenyloxazole per litre. The radioactivities of samples were counted for 5 min, or until 10000 counts had registered, in a Packard Tri-Carb scintillation counter, with automatic external standardization to check that all samples were equally quenched. Quenching was found to be negligible, and counting efficiency was about 80% in all cases.

The error in measurements made by the above procedure is in the range of 5–10%.

RESULTS

ADP-ATP exchange reaction. Ox heart phosphofructokinase catalyses an exchange reaction between [^{14}C]ADP and unlabelled ATP at pH 6.7 and 25°C as shown in Fig. 1.

The absolute dependence of the reaction on the presence of Mg^{2+} ions suggests that the true substrates are the magnesium chelates of the nucleo-

tides. The dependence of the rate of reaction upon substrate concentration at a fixed concentration of the second substrate is hyperbolic within the experimental error (Fig. 2), the K_m for ADP being $20.8 \mu\text{M}$ in the presence of $1000 \mu\text{M}$ -ATP, and the K_m for ATP being $33 \mu\text{M}$ in the presence of $100 \mu\text{M}$ -ADP. There is no sign of inhibition at high ATP/ADP ratios.

The effect of AMP on the exchange reaction was investigated at a high ATP/ADP ratio to allow any putative activation effects to manifest themselves, but as shown in Fig 3(a) AMP is an inhibitor of the reaction. Within the limits of experimental error, citrate has no effect on the rate of the exchange reaction.

The effect of pH on the rate of exchange is shown in Fig. 4. The pK of the reaction is approximately 5.2 under the conditions of the experiment (described in the legend to Fig. 4) and the curve exhibits no extremum.

The maximum observed rate of the ADP-ATP exchange reaction was 0.45 unit/mg of phosphofructokinase, that is about 1% of the maximum observed rate of the forward reaction at this pH (42 units/mg of phosphofructokinase) when measured in the presence of a saturating quantity of AMP.

Fructose 6-phosphate-fructose 1,6-diphosphate exchange reaction. Ox heart phosphofructokinase does not catalyse a direct exchange reaction between fructose 6-phosphate and fructose 1,6-diphosphate in the absence of MgADP^- at pH 8.0, or at pH 6.7, in the presence or absence of magnesium ions. The occurrence of the reaction is absolutely dependent upon the presence of MgADP^- (Fig. 5). The reaction does not proceed in the absence of Mg^{2+}

Fig. 1. Catalysis of [^{14}C]ADP-ATP exchange. ADP and ATP concentrations were 1 mM. Incubation was carried out for 180 min with a phosphofructokinase concentration of $200 \mu\text{g/ml}$. ADP and ATP were separated by chromatography on DEAE-cellulose as described in the Experimental section. ∇ , % transmission at 260 nm; $\circ$, c.p.m. in complete system; $\blacksquare$, c.p.m. in control.

Fig. 2. ADP-ATP exchange. (a) Variation of rate of reaction with ATP concentration at a fixed concentration of $100 \mu M$ -ADP. K_m for ATP is $33 \mu M$, and V_{max} is 2.78 nmol/min . (b) Variation of rate of reaction with ADP concentration at a fixed concentration of 1 mM -ATP. K_m for ADP is $20.8 \mu M$, and V_{max} is 2.38 nmol/min . Units of velocity are nmol/min .

Fig. 3. ADP-ATP exchange. (a) Dixon plot (Dixon & Webb, 1964) for effect of AMP on rate of reaction at fixed concentrations of 1 mM -ATP and $50 \mu M$ -ADP. (b) Dixon plot for effect of citrate on rate of reaction at fixed concentrations of $100 \mu M$ -ADP and $100 \mu M$ -ATP. Incubations were supplemented with an amount of MgSO_4 equivalent to the amount of citrate added. Units of velocity are nmol/min .

ions and AMP does not substitute for ADP in this respect. That MgADP^- is not simply inhibiting the hydrolysis of a phosphorylated-enzyme intermediate is suggested by the observation of Uyeda (1970) that the fructose diphosphatase activity of phosphofructokinase is extremely slight (about 5% of the rate of the reverse reaction at pH 8).

The double-reciprocal plots are linear for all three substrates (Figs. 6 and 7). The exchange reaction proceeds linearly for at least 30 min after the initial addition of enzyme to the reaction mixture. The K_m for fructose 1,6-diphosphate measured in the presence of $10 \mu M$ -fructose 6-phosphate and $10 \mu M$ -ADP was $92.5 \mu M$ and the K_m for fructose 6-phosphate measured in the presence of $50 \mu M$ -fructose 1,6-diphosphate and $10 \mu M$ -ADP was $5.9 \mu M$. The rather low fructose 1,6-diphosphate concentration chosen for the latter measurement was selected to allow any possible nonlinearity in

the double-reciprocal plot to become apparent, fructose 1,6-diphosphate being known to be a kinetic activator of heart phosphofructokinase (E. C. Hulme & K. F. Tipton, unpublished work; Frenkel, 1966). The K_m for ADP measured at a constant concentration of $500 \mu M$ -fructose 1,6-diphosphate is dependent upon the fructose 6-phosphate concentration, being $60 \mu M$ at $100 \mu M$ -fructose 6-phosphate and $133 \mu M$ at $10 \mu M$ -fructose 6-phosphate.

The maximum observed rate of the fructose 6-phosphate-fructose 1,6-diphosphate exchange was 0.725 unit/mg of phosphofructokinase, which is 1.6 times the maximum rate of the ADP-ATP exchange.

The effect of citrate on the exchange is shown in Fig. 8(b). The reaction is subject to strong first-order inhibition by citrate at concentrations comparable with those at which it exerts its kinetic

Fig. 4. Effect of pH on isotope-exchange reactions. Δ , ADP-ATP exchange measured at $500\mu\text{M}$ -ADP and $500\mu\text{M}$ -ATP; $\blacksquare$, fructose 6-phosphate-fructose 1,6-diphosphate exchange measured at $100\mu\text{M}$ -fructose 6-phosphate, $10\mu\text{M}$ -fructose diphosphate, $10\mu\text{M}$ -ADP. The buffer used for this experiment contained 10mm-tris-HCl, 10mm-imidazole-HCl, 100mm-KCl, 10mm-MgSO₄ and 5mm-dithiothreitol. Velocity is expressed as % of maximum observed rate of exchange.

Fig. 5. Catalysis of [¹⁴C]fructose 6-phosphate-fructose 1,6-diphosphate exchange reaction at 1mm-fructose 6-phosphate, $100\mu\text{M}$ -fructose 1,6-diphosphate and $10\mu\text{M}$ -ADP. Incubation was carried out for 90min with $20\mu\text{g}$ of phosphofructokinase/ml. Chromatography on DEAE-cellulose was carried out as described in the Experimental section. $\circ$, Full system; $\blacksquare$, control without enzyme.

effects on the forward reaction. The effect of AMP on the exchange reaction inhibited by citrate is shown in Fig. 8(a). A strong stimulation is seen,

again at concentrations comparable with those at which AMP exerts its kinetic effects.

The effect of pH on the exchange reaction is shown in Fig. 4. The reaction exhibits a sharp optimum at pH 6.8 and tails off very rapidly as the pH drops. The pK of the more acidic group is about 6.2, and that of the more basic groups about 7.4, as assessed by the method of Alberty & Massey (1954).

DISCUSSION

The reaction kinetics of heart phosphofructokinase are known to give families of parallel lines when displayed as plots of reciprocal velocity against reciprocal substrate concentration at a series of fixed concentrations of the other substrate. This kinetic pattern is seen in the case of the forward reaction at pH 8.0, or at pH 6.7 in the presence of saturating quantities of the positive effector AMP, with either fructose 6-phosphate or ATP as the variable substrate (E. C. Hulme & K. F. Tipton, unpublished work) and in the case of the back reaction at pH 8.0 with either fructose 6-phosphate or ADP as the variable substrate (Lorenson & Mansour, 1968).

This pattern corresponds to that generated by the Ping Pong mechanism discussed by Cleland (1963) and is characterized by the involvement of a free modified-enzyme intermediate in the reaction mechanism, the obvious candidate for which in this case is a phosphoryl-enzyme species.

This, although it is compatible with the occurrence of an ADP-ATP exchange, is incompatible with the dependence of the fructose 6-phosphate-fructose 1,6-diphosphate exchange on the presence of MgADP⁻, which implies that the putative $E_{\text{Fru-6-P}}^P$ intermediate releases fructose 6-phosphate slowly if at all in the absence of bound MgADP⁻.

The above conclusion is at variance with the work of Uyeda (1970) on skeletal muscle phosphofructokinase, which is claimed to catalyse a direct fructose 6-phosphate-fructose 1,6-diphosphate exchange, and which exhibits Ping Pong kinetics with ITP and fructose 6-phosphate as substrates, and moreover shows a Ping Pong type of product-inhibition pattern.

The results presented here are best accounted for by a compulsory-order mechanism such as the one shown in Scheme 1. This mechanism accounts for the dependence of the fructose 6-phosphate-fructose 1,6-diphosphate exchange reaction on MgADP⁻, and can account for the occurrence of the ADP-ATP exchange if it is assumed that enzyme-bound ADP can exchange with ADP in free solution.

Fig. 6. Fructose 6-phosphate-fructose 1,6-diphosphate exchange. (a) Variation of reaction velocity with fructose 6-phosphate concentration at fixed concentrations of $10\mu\text{M}$ -fructose 6-phosphate and $10\mu\text{M}$ -ADP. K_m for fructose 1,6-diphosphate is $92.5\mu\text{M}$ and $V_{\max}$ is 0.107 nmol/min . (b) Variation of reaction velocity with fructose 6-phosphate concentration at fixed concentrations of $50\mu\text{M}$ -fructose 1,6-diphosphate and $10\mu\text{M}$ -ADP. K_m for fructose 1,6-diphosphate is $5.9\mu\text{M}$, and $V_{\max}$ is 0.071 nmol/min . Units of velocity are nmol/min .

Fig. 7. Fructose 6-phosphate-fructose 1,6-diphosphate exchange. Effect of ADP on reaction velocity at fixed concentrations of $500\mu\text{M}$ -fructose 1,6-diphosphate, $10\mu\text{M}$ -fructose 6-phosphate; (○) and $100\mu\text{M}$ -fructose 6-phosphate (■). K_m for ADP and $V_{\max}$ are $133\mu\text{M}$ and 0.4 nmol/min and $60\mu\text{M}$ and 4.2 nmol/min respectively. Units of velocity are nmol/min .

The form of the rate equation obtained from a compulsory-order mechanism of this kind is:

$$V = \frac{V_{\max}}{1 + K_m^A/[A] + K_m^B/[B] + K_m^A \cdot K_m^B/[A] \cdot [B]}$$

where K_m^A and K_m^B are the Michaelis constants for A and B, and K_m^A is the apparent binding constant for the first substrate bound (i.e. A), K_m^B is the

binding constant that would be measured in a direct-binding experiment.

In general, the above equation will give convergent double-reciprocal plots. The $1/[S]$ co-ordinate of the intersection point with A as the variable substrate is $-1/K_m^A$, and with B as the variable substrate is $-K_m^A/K_m^B \cdot K_m^A$. The $1/V$ co-ordinate of the intersection point is the same in both cases, and is equal to $1 - K_m^A/K_m^A$. The nonconvergence of the double reciprocal plots thus requires that $K_m^{\text{ATP}'}$ and $K_m^{\text{Fru-6-P}} \cdot K_m^{\text{ATP}'}/K_m^{\text{ATP}}$ should be small compared with the substrate concentrations at which kinetic measurements can be feasibly made. By using present techniques, it is difficult to make kinetic measurements at substrate concentrations less than $10\mu\text{M}$ for this enzyme and, since the $K_m^{\text{ATP}'}$ is $2\mu\text{M}$ (Lorenson & Mansour, 1969), and the K_m values for MgATP^{2-} and fructose 6-phosphate are 40 and $112\mu\text{M}$ respectively (E. C. Hulme & K. F. Tipton, unpublished work), convergence of the double-reciprocal plots is not detectable.

The K_m for ATP is directly proportional to the velocity constant for the breakdown of the complex

EFru-6-P (Scheme 1). This complex must thus be

broken down relatively rapidly, since $K_m^{\text{Fru-6-P}}/K_m^{\text{ATP}} \cdot K_m^{\text{ATP}'}$ is required to be small. Mechanisms incorporating this feature have been discussed by Theorell & Chance (1951) and characteristically give a Ping Pong type of inhibition pattern, which is consistent with the kinetic studies of Uyeda (1970).

Fig. 8. Fructose 6-phosphate-fructose 1,6-diphosphate exchange. (a) Effect of AMP on rate of exchange at constant concentrations of $5\mu\text{M}$ -fructose 6-phosphate, $50\mu\text{M}$ -fructose 1,6-diphosphate, 10mM -ADP and 10mM -citrate. The incubation mixture was supplemented with 10mM - MgSO_4 in this case. (b) Dixon plot for effect of citrate on rate of exchange at constant concentrations of $5\mu\text{M}$ -fructose 6-phosphate, $10\mu\text{M}$ -ADP and $50\mu\text{M}$ -fructose 1,6-diphosphate. The incubation mixture was supplemented with an amount of MgSO_4 equivalent to the amount of citrate added.

Scheme 1. Scheme proposed for the basic reaction mechanism of ox heart phosphofructokinase.

Influence of pH and effectors. The binding studies of Lorensen & Mansour (1969) together with the molecular-weight studies by Mansour & Ahlfors (1967) on heart phosphofructokinase and by Paetkau & Lardy (1966) on muscle phosphofructokinase suggest that the smallest fully active form of heart phosphofructokinase has a molecular weight of 360 000, consisting of four identical subunits of molecular weight approx. 90 000, which are themselves probably composed of two regulatory and two catalytic subunits of molecular weight about 25 000. Incubation of a dilute solution of the enzyme at pH 6.5 favours dissociation of the molecular weight 360 000 form into two subunits of molecular weight 180 000, whose specific activity amounts to only a few per cent of that of the native enzyme.

The classical shape and the relatively low pK of the pH-activity curve of the ADP-ATP exchange reaction suggests that this half-reaction is independent of the state of association of the protein, and may be a function of the 90 000 molecular-weight subunits alone. The pK of the reaction (5.2) corresponds closely to that of the ionization of the magnesium complexes of ATP and ADP (Phillips, 1966), which may imply that the species MgATP^{2-} and the corresponding ADP species are the true

substrates of the exchange. The absence of any marked inhibition by high citrate or ATP concentrations suggests that this half-reaction is not subject to control by effectors.

The inhibition of the ADP-ATP exchange by AMP can probably be ascribed to competition with MgADP^- for binding, and is unlikely to affect the kinetics of the overall reaction.

However, the very abrupt tail-off of the fructose 6-phosphate-fructose 1,6-diphosphate exchange reaction as the pH falls below 7.0 suggests strongly that its occurrence is dependent on the integrity of the 360 000-molecular-weight form of the enzyme. There is a close correspondence between the acid limb of this curve, and the curve obtained by pre-incubating the enzyme at a series of different pH values and then assaying it at pH 8.0 (E. C. Hulme, unpublished work; Lorensen & Mansour, 1968), suggesting that one is effectively determining a titration curve for the dissociation of the enzyme. The pK of this process is about 6.2. The optimum pH of the exchange is about 0.8 pH unit lower than that of the forward reaction (E. C. Hulme, unpublished work).

The observation of citrate inhibition and AMP activation of the exchange strongly suggests that this is the half-reaction that is subject to the effector control characteristic of phosphofructokinase. This control must be exerted by modulation of the binding affinity of the catalytic site for fructose 6-phosphate in the forward direction, or for fructose 1,6-diphosphate in the reverse direction, or on the rate of transfer of the phosphate group. The observa-

tions that have been presented above are in good agreement with the model of phosphofructokinase put forward by the authors on the basis of kinetic experiments (Hulme & Tipton, 1971).

The authors thank Professor F. G. Young for the provision of laboratory space. This work was performed during the tenure of a Science Research Council research studentship by E.C.H.

REFERENCES

- Alberty, R. A. & Massey, V. (1954). *Biochim. biophys. Acta*, **13**, 317.
- Cleland, W. W. (1963). *Biochim. biophys. Acta*, **67**, 104.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 329. London: Longmans Green and Co. Ltd.
- Frenkel, R. (1966). *Archs Biochem. Biophys.* **25**, 166.
- Hulme, E. C. & Tipton, K. F. (1971). *FEBS Lett.* (in the Press).
- Layzer, R. B., Rowland, L. P. & Banks, W. J. (1969). *J. biol. Chem.* **244**, 3823.
- Lorenson, M. Y. & Mansour, T. E. (1968). *J. biol. Chem.* **243**, 4677.
- Lorenson, M. Y. & Mansour, T. E. (1969). *J. biol. Chem.* **244**, 6420.
- Mansour, T. E. & Ahlfors, C. E. (1967). *J. biol. Chem.* **243**, 2523.
- Paetkau, V. & Lardy, M. A. (1966). *J. biol. Chem.* **242**, 2035.
- Phillips, R. C. (1966). *Chem. Rev.* **66**, 501.
- Theorell, H. & Chance, B. (1951). *Acta chem. scand.* **5**, 1127.
- Uyeda, K. (1970). *J. biol. Chem.* **245**, 2268.