Eur. J. Biochem. 134, 283 – 288 (1983)
© FEBS 1983

Isotope-Exchange Evidence that Glucose 6-Phosphate Inhibits Rat-Muscle Hexokinase II at an Allosteric Site

Mary GREGORIOU, Ian P. TRAYER, and Athel CORNISH-BOWDEN
Department of Biochemistry, University of Birmingham

(Received January 13 / April 1, 1983) – EJB 83 0028

The flux ratio for hexokinase type II from rat muscle, i.e. the rate of conversion of glucose 6-phosphate molecules into ATP molecules divided by the simultaneous rate of conversion of glucose 6-phosphate molecules into glucose molecules, increases with the MgATP concentration but is independent of the glucose concentration. This behaviour requires that glucose must bind before MgATP when the reaction is proceeding in the normal physiological direction, i.e. phosphorylation of glucose. Although at low non-inhibitory glucose 6-phosphate concentrations the flux ratio increases linearly with the MgATP concentration, the dependence becomes non-linear, with a slope that increases with the MgATP concentration, at glucose 6-phosphate concentrations above 1 mM. This behaviour does not permit glucose 6-phosphate to act only as a normal product inhibitor. Instead, it seems to require glucose 6-phosphate to act as an allosteric inhibitor and for a second site for binding of MgATP to exist. Measurements of the flux from ATP to glucose 6-phosphate and to ADP showed no dependence of the flux ratio on the concentrations of either glucose 6-phosphate or ADP. This result does not permit the order of product-release steps in this direction to be determined, but shows that the second product is released virtually instantaneously after the first.

There has been controversy about the mechanism of action of the mammalian hexokinase isoenzymes since they were first discovered. The early arguments about the existence of a phosphoenzyme intermediate have now apparently been resolved in favour of a mechanism that proceeds through a ternary enzyme-glucose-ATP complex [1], but other disputes continue. There has been doubt about whether the ternary complex can be formed by binding of substrates in a random order or whether glucose must bind before ATP. There is also still argument about whether glucose 6-phosphate, a potent inhibitor of hexokinases I and II, the predominant isoenzymes in brain and muscle respectively, acts simply as a product or whether it binds at an allosteric site. The allosteric interpretation was proposed (in different terminology) long ago by Crane and Sols [2]; it was recently reaffirmed for hexokinase I [3], but again challenged even more recently [4].

The flux-ratio method [5] provides a less ambiguous way of determining the order of binding of substrates than the more commonly used product-inhibition method, and Colowick [1] suggested that it would be helpful for resolving some of the conflicts over the mammalian hexokinases. We have recently applied it to the liver isoenzyme, hexokinase IV (also known as glucokinase), and have shown that that enzyme follows a preferred-order mechanism with glucose binding first [6]. We have now carried out flux-ratio measurements with hexokinase II purified to homogeneity and high specific activity from rat muscle. The results appear to resolve both the question of the order of binding of substrates and the nature of the inhibition by glucose 6-phosphate. We have given a preliminary account of these results previously [7].

Abbreviations. F(Q → A), flux from Q to A. The following are used in algebraic expressions: Glc, glucose; Glc6*P*, glucose 6-phosphate.

Enzymes. Hexokinase, ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1); hexokinase type IV is also known as glucokinase, ATP: D-glucose 6-phosphotransferase (EC 2.7.1.2).

MATERIALS AND METHODS

Chemicals

D-[U-^{14}C]Glucose (260 Ci/mol), adenosine 5′-[γ-^{32}P]triphosphate (20 Ci/mmol), [8-^{3}H]adenosine 5′-triphosphate (21 Ci/mol) and [U-^{14}C]adenosine 5′-diphosphate (500 – 600 Ci/mol) were obtained from Amersham International Ltd (Amersham, Bucks, UK). Adenosine 5′-diphosphate (dimonocyclohexylammonium salt) grade VI, adenosine 5′-triphosphate (disodium salt), D-glucose 6-phosphate and imidazole, grade III, were purchased from Sigma Chemical Co. (Poole, Dorset, UK) and DEAE-cellulose paper (DE81) from Whatman Ltd (Maidstone, Kent, UK).

Purification of Rat-Skeletal-Muscle Hexokinase II

Hexokinase II was purified to homogeneity by the method of Holroyde and Trayer [8]. The key step in the purification was affinity chromatography on the *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose derivative of Sepharose-4B. The purified enzyme was found to be at least 95% pure as judged by two-dimensional polyacrylamide gel electrophoresis and isoelectric focussing, and had a specific activity of 3300 kat/kg.

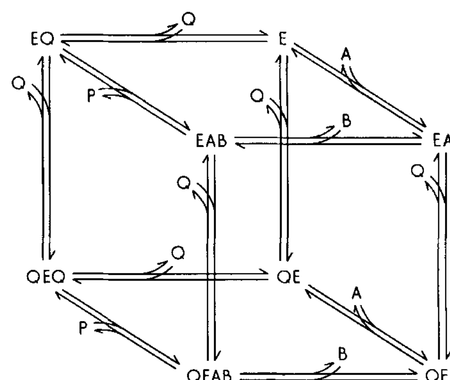
Preparation of Radioactively Labelled Glucose 6-Phosphate

D-[^{14}C]Glucose 6-phosphate and D-glucose 6-[^{32}P]phosphate were prepared from D-[U-^{14}C]glucose and adenosine 5′-[γ-^{32}P]triphosphate as described previously [6] and purified by chromatography on DEAE-cellulose.

Flux Measurements

To measure fluxes of substrates to products, in either direction, suitably radioactively labelled substrates were in-

cubated with hexokinase and at time intervals aliquots from the reaction mixture were chromatographed on strips of DEAE-cellulose paper (DE81) for separation of the reaction components, as described in detail previously [6]. The radioactive content of products was determined by liquid scintillation counting and the initial rates of appearance of label in products were determined from progress curves. All reactions were carried out in 50-mM imidazole nitrate buffer, pH 6.5, containing 1-mM dithiothreitol, 1-mM excess Mg^{2+} over the total adenine nucleotide concentration and 100-mM KCl. An excess of 1-mM Mg^{2+} is sufficient to ensure that ATP and ADP exist in a large and nearly constant proportion as their 1:1 complexes with Mg^{2+} [9].



Scheme 1. *Model for allosteric inhibition.* In this model the second product Q is assumed to act not only as a product, but also as an allosteric inhibitor able to produce a form of the enzyme QE that can, in principle, undergo all of the reactions that occur in the normal reaction pathway. Although various special cases of this model are capable of predicting a downward curving dependence of the flux ratio $F(\mathrm{Q} \rightarrow \mathrm{B})/F(\mathrm{Q} \rightarrow \mathrm{A})$ on the concentration of the second substrate B, as described in the text, it appears impossible with this type of mechanism to obtain an upward curvature of the sort seen in Fig. 2

THEORY

Measurement of Flux Ratios

Regardless of the magnitude and direction of the bulk chemical reaction, the rate at which individual molecules of a product, e. g. glucose 6-phosphate, are being converted into molecules of a substrate, e. g. ATP, can be measured by means of a suitably labelled product, glucose 6-[^{32}P]phosphate in this case. This rate is called the flux from glucose 6-phosphate to ATP, and is symbolized in this paper as $F(\mathrm{Glc}6P \rightarrow \mathrm{ATP})$. If $F(\mathrm{Glc}6P \rightarrow \mathrm{Glc})$, the corresponding flux from glucose 6-phosphate to glucose, is measured simultaneously (by means of [^{14}C]glucose 6-phosphate), the flux ratio $F(\mathrm{Glc}6P \rightarrow \mathrm{ATP})/F(\mathrm{Glc}6P \rightarrow \mathrm{Glc})$ provides information about the order of binding of glucose and ATP. This information is less ambiguous than that given by more widely used techniques, such as product inhibition, because it is qualitatively unaffected by such complexities as dead-end inhibition, isomerization of the free enzyme, and a random order of product release. The flux-ratio method was introduced by Britton [5] and subsequently extended by Britton and Dann [10]. We have recently described its application to rat-liver hexokinase IV (glucokinase) [6], and have also given a simple account of the theory [11]. Here we shall only discuss certain special cases that did not need to be considered in the previous studies.

General Mechanism for Allosteric Inhibition

A general mechanism for allosteric inhibition (or activation) of a two-substrate two-product reaction, with Q acting both as a product and also as an allosteric inhibitor, is shown in Scheme 1. The two functions of Q are considered to be distinct, so that the species QE, in which it is bound to the allosteric site, is not the same as EQ, in which Q is bound at the catalytic site. If no simplifying assumptions are made the expressions for the fluxes from Q to A and from Q to B in Scheme 1 are too complex to be manageable, so we shall examine three special cases.

Allosteric Inhibition at Equilibrium

If the binding of Q at the allosteric site is at equilibrium in all states of the enzyme, all of the 'vertical' steps in Scheme 1 are very fast. Each pair of species at equilibrium can then be treated as a single species in deriving rate equations [12], with rate 'constants' that depend on the state of the equilibrium. The rate 'constants' are all functions of [Q], but not of any of the other reactant concentrations, and so the flux ratio depends on [A] and [B] in the same way as for a compulsory-order mechanism without allosteric effects. In other words $F(\mathrm{Q} \rightarrow \mathrm{B})/F(\mathrm{Q} \rightarrow \mathrm{A})$ is independent of [A] and a linear function of [B] [6, 10, 11].

Allosteric Inhibition Very Slow

The other extreme is to assume that all 'vertical' steps in Scheme 1 are so slow that flux through them can be ignored in deriving the flux expressions. The flux from Q to A, for example, is then simply the sum of the fluxes in the 'upper' and 'lower' levels of the Scheme. As B occurs twice in the pathway between Q and A we should expect from consideration of the method of King and Altman [13] that the expression for $F(\mathrm{Q} \rightarrow \mathrm{A})$ would contain terms in $[\mathrm{B}]^2$, and detailed algebraic analysis confirms this and shows that a plot of the flux ratio $F(\mathrm{Q} \rightarrow \mathrm{B})/F(\mathrm{Q} \rightarrow \mathrm{A})$ against [B] is curved. If the equation for this curve is differentiated twice with respect to [B], the resulting second derivative proves to be negative, i.e. the slope of the curve decreases as [B] increases.

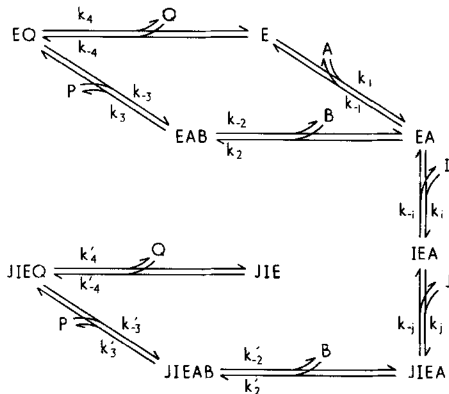
Allosteric Inhibition Only with First Substrate Bound

The product inhibition characteristics of hexokinase II (see Discussion) suggest that we ought to consider a mechanism in which the allosteric inhibitor Q binds only to EA and EAB, so that QE and QEQ do not exist. In this case it is feasible to derive an expression for the flux ratio withouth any assumption that particular steps are very fast or very slow. It is found that the plot of flux ratio against [B] is again curved, and again the curvature is downward, i. e. the slope decreases as [B] increases.

Similar characteristics apply to all special cases of Scheme 1 that we have studied, and suggest that in general the release of a substrate B in two (or more) parallel steps before A will often generate a non-linear dependence of $F(\mathrm{Q} \rightarrow \mathrm{B})/F(\mathrm{Q} \rightarrow \mathrm{A})$ on [B] but the curvature will always be downward.

Allosteric Inhibition Enhanced by the Second Substrate

It would appear from the considerations discussed that an upward curving plot of $F(\mathrm{Q} \rightarrow \mathrm{B})/F(\mathrm{Q} \rightarrow \mathrm{A})$ against [B] cannot be generated by mechanisms in which the multiple steps in



Scheme 2. *Proposed mechanism for hexokinase II.* This model differs from that shown in Scheme 1 by requiring a molecule of the second substrate B to bind to the allosterically inhibited enzyme QEA at a second allosteric site to give a form of the enzyme BQEA that is capable of undergoing all of the catalytic reactions apart from binding and release of the first substrate A. In deriving flux expressions it is important to distinguish between the molecules of B and Q bound at the catalytic site, which can be interconverted, and the molecules of B and Q bound at the allosteric site, which cannot. For this reason, the latter are represented as J and I respectively in the scheme and also in the derivation in the text. In the model proposed for hexokinase II, A corresponds to glucose, B and J to ATP, P to ADP, and Q and I to glucose 6-phosphate

which B is released occur only in parallel. Instead, it appears necessary for two (or more) molecules of B to be released in series before a molecule of A can be released. The simplest way in which we can conceive of this happening in the inhibited, but not in the uninhibited, form of an enzyme is illustrated in Scheme 2. Two points about this scheme should be noted. It assumes that the chemical conversion of Q into B can occur when the allosteric site is occupied, but conversion into A cannot, and it assumes that allosteric binding of a second molecule of B is also required.

In deriving flux expressions for Scheme 2 it is necessary to distinguish between molecules of Q and B at the active site, which can be interconverted, and molecules of Q and B at the allosteric site, which cannot. To avoid confusion, therefore, Q and B acting allosterically will be represented during the derivation of the expression for the flux ratio simply as inhibitors I and J respectively.

The fluxes from Q to B and from Q to A may be derived by the methods described previously [6], and are as follows:

$$F(\mathrm{Q} \rightarrow \mathrm{B}) = \frac{v_{-2}v_{-5}}{v_{-2}+v_5} + \frac{v'_{-2}v'_{-5}}{v'_{-2}+v'_5} \quad (1)$$

$$F(\mathrm{Q} \rightarrow \mathrm{A}) = \frac{v_{-1}\left[\dfrac{v_{-2}v_{-5}}{v_{-2}+v_5} + \dfrac{v_{-ij}v'_2\, v'_{-5}/(v'_{-2}+v'_5)}{v_{-ij}+v'_2v'_5/(v'_{-2}+v'_5)}\right]}{v_{-1} + \dfrac{v_2v_5}{v_{-2}+v_5} + \dfrac{v_{ij}v'_2\, v'_5/(v'_{-2}+v'_5)}{v_{-ij}+v'_2v'_5/(v'_{-2}+v'_5)}} \quad (2)$$

In these expressions, each v represents the flux through the step whose rate constant has the same subscript, i.e. $v_{-2} = k_{-2}$ [EAB], $v_2 = k_2$[EA] [B], etc., the rate constants themselves being defined in Scheme 2. In addition, the expressions are simplified by writing $v_5 = v_3v_4/(v_{-3}+v_4)$, $v_{-5} = v_{-3}v_{-4}/(v_{-3}+v_4)$, with v'_5 and v'_{-5} defined correspondingly (i.e. the same expressions with primes throughout). As v_{-3} and v'_{-3} are dependent on [P], these simplifications obscure the dependence of the fluxes on [P], but as long as [P] is held constant this presents no difficulties, and no additional assumptions are implied by them. For the same reason, v_{ij} and v_{-ij} are written instead of $v_iv_j/(v_{-i}+v_j)$ and $v_{-i}v_{-j}/(v_{-i}+v_j)$ respectively.

If Eqn (1) is divided by Eqn (2) and each v is replaced by the corresponding expression in terms of rate constants and concentrations of enzyme forms and reactants, the resulting expression for the flux ratio is extremely complicated and the concentrations of E and JIE do not cancel. It can, however, be greatly simplified by taking account of the fact that the dependence of the flux ratio on [B] is normally measured in the absence of A, and by expressing [E] in terms of [JIE], derived in the normal way [13] with [A] = 0, as follows:

$$[\mathrm{E}] = \frac{[\mathrm{JIE}]\,\{k_{-1}(k_{-2}+k_5) + k_2k_5[\mathrm{B}]\}\,k_{-ij}k'_{-2}k'_{-5}}{k_{-2}k_{-5}k_{ij}k'_2k'_5\,[\mathrm{B}]\,[\mathrm{I}]\,[\mathrm{J}]}. \quad (3)$$

With these substitutions extensive cancellation becomes possible in the flux expression, which then takes the following form:

$$\frac{F(\mathrm{Q}\rightarrow\mathrm{B})}{F(\mathrm{Q}\rightarrow\mathrm{A})} = 1 + \frac{k_2k_5[\mathrm{B}]}{k_{-1}(k_{-2}+k_5)} + \frac{k_{ij}k'_2k'_5[\mathrm{B}][\mathrm{I}][\mathrm{J}]}{k_{-1}(k'_{-2}+k'_5)k_{-ij}}. \quad (4)$$

At it stands, Eqn (4) shows the flux ratio to be a linear function of [B]. However, if we replace I and J by Q and B respectively, as in the original form of Scheme 2, the dependence of the flux ratio on [B] becomes obvious. At low [Q], there is a linear dependence with unit intercept on the ordinate, but at high [Q] there is a parabolic dependence, again with unit intercept. As all of the rate constants and concentrations must be zero or positive, any curvature must be upwards, i.e. the slope must increase with [B].

RESULTS

Rates of Isotope Exchange at Chemical Equilibrium

At saturating concentrations of substrates and products, present at chemical equilibrium, the rates of isotope exchange ATP ⇄ ADP, ATP ⇄ glucose 6-phosphate, and glucose ⇄ glucose 6-phosphate were very similar. This contrasts with the much more rapid exchange between ATP and ADP than between glucose and glucose 6-phosphate found with hexokinase IV [6], and suggests that for hexokinase II the chemical step is rate-limiting. The dependence of the routes of isotope exchange on the degree of saturation of the enzyme was difficult to investigate at chemical equilibrium because of the unfavourable magnitudes of the equilibrium constant and the kinetic parameters ([ADP] [Glc6*P*]/[ATP] [Glc] = 234 at equilibrium; K_m^{Glc} = 0.2 mM; K_m^{ATP} = 0.8 mM; K_i^{ADP} = 1.6 mM with respect to ATP; K_i^{Glc6P} = 0.075 mM with respect to glucose). It was more convenient, therefore, to study the reaction under non-equilibrium conditions.

Variation of the Flux Ratio with Glucose and ATP Concentrations

In the absence of ATP the ratio *F*(Glc6*P* → Glc)/*F*(Glc6*P* → ATP) was found to be independent of the glucose concentration and equal to unity over the whole concentration

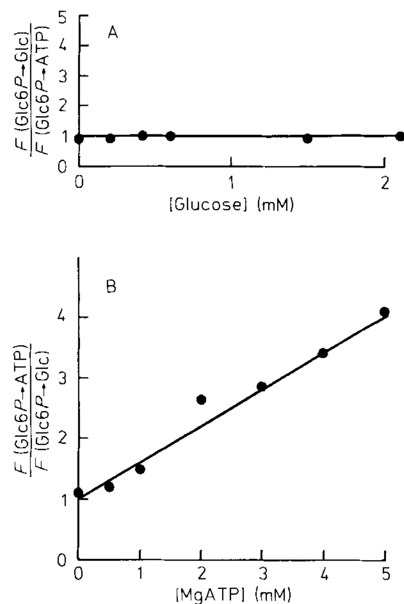


Fig. 1. *Flux ratios at non-inhibitory concentrations of glucose 6-phosphate.* The individual fluxes $F(\text{Glc6}P \rightarrow \text{Glc})$ and $F(\text{Glc6}P \rightarrow \text{ATP})$ were measured simultaneously in the same reaction mixtures from the rates of appearance of ^{14}C-labelled glucose and ^{32}P-labelled ATP from doubly labelled glucose 6-phosphate (20 µCi ^{14}C and 6.7 µCi ^{32}P), in the presence of 5-mM MgADP and 0.085-mM glucose 6-phosphate. The flux ratio $F(\text{Glc6}P \rightarrow \text{Glc})/F(\text{Glc6}P \rightarrow \text{ATP})$ was constant at unity when measured at increasing concentrations of glucose in the absence of ATP (A), but the reciprocal flux ratio $F(\text{Glc6}P \rightarrow \text{ATP})/F(\text{Glc6}P \rightarrow \text{Glc})$ showed a linear dependence on the concentration of ATP when measured at increasing ATP concentration in the absence of glucose (B). This behaviour is characteristic of a compulsory-order mechanism in which glucose binds first.

range studied, from zero to 10 times K_m (Fig. 1 A). This behaviour indicates a highly preferred order of substrate binding, with glucose binding to the free enzyme. In the absence of glucose, and at glucose 6-phosphate concentrations low enough for inhibition to be negligible, the ratio $F(\text{Glc6}P \rightarrow \text{ATP})/F(\text{Glc6}P \rightarrow \text{Glc})$ showed a linear dependence on the ATP concentration, increasing from a value of unity in the absence of ATP (Fig. 1 B). This is characteristic of a compulsory-order mechanism in which ATP is second substrate, and is very similar to the behaviour of hexokinase IV [6].

When the flux ratio was measured at inhibitory concentrations of glucose 6-phosphate, a novel kind of behaviour was observed. The flux ratio increased with the concentration of glucose 6-phosphate, and the linear dependence on the concentration of ATP became curved, with a slope that increased with the ATP concentration (Fig. 2).

The observed behaviour is inconsistent with any mechanism in which glucose 6-phosphate acts only as a product, because the concentration of glucose 6-phosphate must always cancel from the expression for $F(\text{Glc6}P \rightarrow \text{ATP})/F(\text{Glc6}P \rightarrow \text{Glc})$ for any mechanism in which there is only one step in which glucose 6-phosphate binds on the transfer route. It is also inconsistent with all of the special cases of Scheme 1 that we have considered, and we believe it to be inconsistent with the general case of Scheme 1, though the extreme complexity of the algebra has prevented a rigorous proof of this. Thus the effects of glucose 6-phosphate and ATP seem to rule out not only normal product inhibition as the cause of the potent inhibition of hexokinase II by glucose 6-phosphate, but also any con-

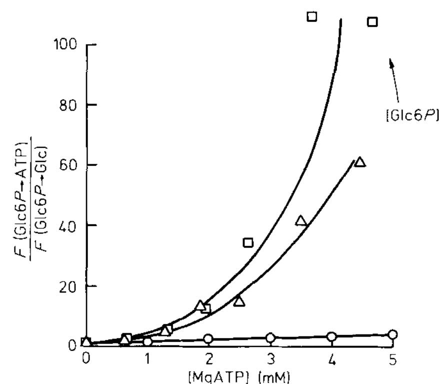


Fig. 2. *Effect of inhibition by glucose 6-phosphate on the dependence of the flux ratio on the ATP concentration.* The concentrations of glucose 6-phosphate were 0.085 mM (○), 2.85 mM (△) and 3.00 mM (□), with the following specific activities per µmol of glucose 6-phosphate: 2.6 mCi ^{32}P, 7.5 mCi ^{14}C (○); 19.5 µCi ^{32}P, 21.8 µCi ^{14}C; (△); 18.5 µCi ^{32}P, 20.7 µCi ^{14}C (□). Other experimental conditions were as in Fig. 1 (B). The dependence of the flux ratio on the concentration of glucose 6-phosphate is not consistent with any mechanism in which glucose 6-phosphate acts only as a substrate (i. e. only as a product of the reaction proceeding in the normal physiological direction). The upward curvature appears to require a reaction pathway to exist in which two molecules of ATP must be released before any glucose can be released

ventional kind of allosteric inhibition. Instead, the data appear to require a mechanism in which the inhibited enzyme is capable of catalysing exchange between glucose 6-phosphate and ATP, but not between glucose 6-phosphate and glucose, and release of glucose from the inhibited enzyme requires not only release of glucose 6-phosphate, but also release of two molecules of ATP, one of which is presumably bound at an allosteric site.

Variation of the Flux Ratio with Glucose 6-Phosphate and ADP Concentrations

The order of dissociation of ADP and glucose 6-phosphate from the ternary complex was investigated by studying the dependence of the flux ratio $F(\text{ATP} \rightarrow \text{ADP})/F(\text{ATP} \rightarrow \text{Glc6}P)$ on the concentration of glucose 6-phosphate (in the absence of ADP) and ADP (in the absence of glucose 6-phosphate). In both experiments the flux ratio remained constant at unity over the whole concentration ranges studied (Fig. 3). This contrasts with the behaviour of hexokinase IV [6], for which there was a linear dependence of the flux ratio on the concentration of ADP but no dependence on that of glucose 6-phosphate, indicating release of ADP before glucose 6-phosphate for that isoenzyme. In the present case, the lack of dependence of flux ratio on either concentration indicates that whichever product is released first the second is released so quickly that increasing the concentration of the first product cannot compete effectively with the second release step.

Attempts to Trap Binary Complexes

Attempts were made to trap enzyme-ADP and enzyme–glucose-6-phosphate complexes under steady-state conditions by detecting transfer of radioactive label from products to substrates while the unlabelled reaction was proceeding from substrates to products. The experiment was done by incubating the enzyme with glucose and ATP in the presence of labelled product, either ADP or glucose 6-phosphate. In no case could

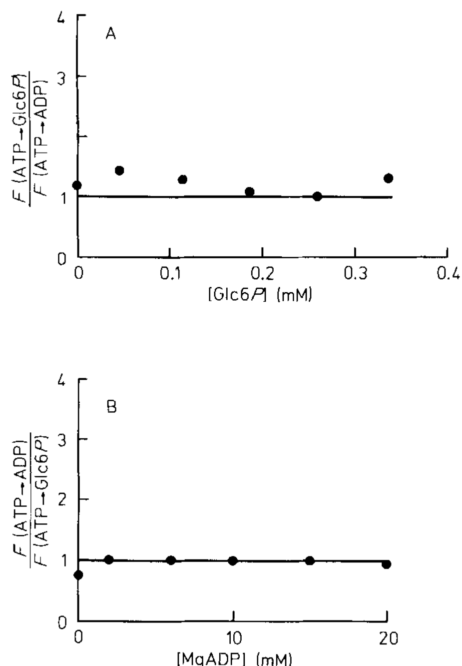


Fig. 3. *Flux ratios for flux from ATP to products.* The reactions were carried out in the presence of 1.65-mM MgATP (50.5 µCi [8-^{3}H]ATP/µmol and 20.2 µCi [γ-^{32}P]ATP/µmol) and 3.3-mM glucose. The enzyme concentration was chosen so that no more than 0.01-mM glucose 6-phosphate accumulated during the 5 min of each reaction. Under the conditions used no adenosinetriphosphatase activity could be detected and so any ^{3}H-labelled ADP produced was the result of kinase activity. The flux ratio F(ATP → Glc6P)/F(ATP → ADP) was constant at a value of unity whether measured at increasing concentrations of glucose 6-phosphate in the absence of ADP (A) or at increasing concentrations of ADP in the absence of glucose 6-phosphate (B). This behaviour does not define an order of product release, but indicates that the second product-release step follows so rapidly on the first that the two are virtually concerted

any radioactivity be detected in either substrate: this is consistent with the result of the flux-ratio experiment, as it shows that any binary enzyme-product complex that occurs when the reaction is proceeding forwards dissociation so rapidly that it cannot be trapped.

DISCUSSION

The flux-ratio measurements provide for the first time unambiguous evidence that glucose and ATP bind to hexokinase II in compulsory order, and that the potent inhibition of the enzyme by glucose 6-phosphate is not due to normal inhibition by a product of the reaction. The dependence of the flux ratio for flux from glucose 6-phosphate to substrates on the concentration of glucose 6-phosphate (Fig. 2) is inconsistent with any mechanism in which glucose 6-phosphate binds to the enzyme in one step only. Moreover, the form of the dependence of the flux ratio on the concentration of ATP in the presence of inhibitory concentrations of glucose 6-phosphate appears to be inconsistent with all mechanisms in which ATP-binding steps occur only in parallel. Release of ATP in two (or more) steps in series thus requires a mechanism at least as complex as that shown in Scheme 2, though the real mechanism may of course be still more complex.

The flux-ratio measurements for flux from ATP to ADP and glucose 6-phosphate (Fig. 3), together with the failure to trap any binary enzyme-product complexes, indicate that the second product-release step is very fast, but they do not allow determination of which product is released first. Analogy with hexokinase IV (glucokinase) suggests that ADP is released before glucose 6-phosphate [6]. Although there are substantial kinetic differences between hexokinase IV and the other mammalian isoenzymes, there is ample evidence from structural, chemical and stereochemical studies to indicate a basic unity of mechanism [14–16]. In addition, the close similarity between hexokinases I and II, which includes kinetic as well as structural similarity [1], suggests that hexokinase I also follows a compulsory-order mechanism with allosteric inhibition by glucose 6-phosphate. This is important for the present discussion, because much of the arguments over the mechanism of the mammalian hexokinases has been conducted in the context of the brain enzyme, hexokinase I. It is thus convenient to discuss previous work in relation to both hexokinases I and II, especially as we are not aware of any dissent from the view that they are mechanistically equivalent.

Although there have been claims that substrates bind in random order to hexokinases I and II [17–19], Ricard and co-workers [20] have shown that the earlier claims were based on faulty reasoning from inadequate evidence. Subsequently Lueck and Fromm [18] presented data that they believe showed both N-acetylglucosamine (a glucose analogue) and ATP^{4-} (an $MgATP^{2-}$ analogue) to act as competitive inhibitors with respect to the analogous substrates and as 'non-competitive' (i.e. mixed) inhibitors with respect to the non-analogous substrates. Examination of their data, however, suggests that ATP^{4-} is virtually uncompetitive with respect to glucose (Fig. 4 of [18]), exactly as one would expect for an analogue of the second substrate in a compulsory-order mechanism. The slight deviation from uncompetitive inhibition can probably be accounted for in terms of the impossibility of maintaining an exactly constant pH and Mg^{2+} concentration while varying both ATP^{4-} and $MgATP^{2-}$ concentrations independently [9].

It follows that the results of the isotope exchange experiments described in the present paper are not in conflict with any clear evidence for a random-order mechanism. The nature of the inhibition by glucose 6-phosphate presents a greater problem, however, because Fromm and his co-workers have continued to maintain that this is due to ordinary product inhibition, with glucose 6-phosphate competing with ATP for the γ-phosphate subsite in the catalytic site [4]. The most recent arguments are based on the assertion that the reaction catalysed by hexokinase I obeys 'classical Michaelis-Menten kinetics' when studied in the reverse direction [4]. It is difficult, however, to reconcile this assertion with the evidence adduced in its favour: although Fig. 1 of [4] is presented as a series of straight-line plots of $1/v$ against $1/[\mathrm{Glc6}P]$, the experimental points do not lie on the lines drawn; that this represents a systematic failure of the Michaelis-Menten equation, rather than, for example, poor experimental precision, is clear from the fact that curvature in the same direction is evident in each of the five plots given. Accordingly, we do not believe there is evidence against the proposal that glucose 6-phosphate binds at an allosteric site.

We must also consider whether our interpretation of the flux-ratio experiments is consistent with the product-inhibition data reported by Lueck and Fromm [18] for hexokinase II. They found that glucose 6-phosphate acted as a mixed inhibitor with respect to both glucose and ATP, but the inhibition was almost purely competitive in the case of ATP. If glucose 6-phosphate acted only as a competitor for the ATP site, we should expect it to be uncompetitive with respect to glucose but

competitive with respect to ATP; whereas if it acted only as a product it would be competitive with respect to glucose but a mixed inhibitor with respect to ATP. In our proposals (Scheme 2) it acts predominantly as an allosteric inhibitor, but as it is a product of the reaction it must be capable of acting as a product inhibitor to some degree, and so intermediate behaviour, mixed with respect to both substrates but almost competitive in the case of ATP, is entirely reasonable. As Scheme 2 requires not only glucose 6-phosphate but also ATP to bind allosterically, the predicted inhibition behaviour is somewhat more complex than this, with more uncompetitive character, but only if the allosteric binding of ATP occurs to a significant degree under ordinary steady-state conditions: the fact that no substrate inhibition by ATP is evident in the results of Lueck and Fromm [18] suggests that is does not.

In this discussion we have not considered the possibility that the complex behaviour observed might be due to interactions between multiple active sites on the enzyme. This is because hexokinase II is not only monomeric under a wide variety of conditions, including those used in the assay [8], but also shows complete loss of catalytic activity on reacting with 1 mol of the affinity label *N*-bromoacetylglucosamine/mol of enzyme [24]. Although it is not impossible to account for this behaviour in terms of multiple active sites, for example if reaction with *N*-bromoacetylglucosamine caused complete loss of activity, including the capacity to react with additional molecules of *N*-bromoacetylglucosamine, at every active site on the labelled molecule, the simplest and most plausible interpretation is to suppose that hexokinase II is a monomeric enzyme with only one active site per molecule.

The results with hexokinase II have again illustrated the great power of the flux-ratio method. Nearly thirty years of conventional steady-state experiments have failed to resolve the controversies over the order of binding of substrates to the mammalian hexokinases or the nature of the potent inhibition by glucose 6-phosphate. The flux-ratio measurements have, by contrast, left no doubt that glucose binds before ATP, and they have shown that glucose 6-phosphate cannot be acting solely as a product. They have also excluded the conventional type of allosteric mechanism represented by Scheme 1, because this seems incapable of explaining the parabolic dependence of flux ratio on the concentration of ATP at inhibitory concentrations of glucose 6-phosphate. Instead, we have been forced to consider the type of mechanism shown in Scheme 2. Although the suggestion of an allosteric binding site for ATP is novel, it is not absurd in relation to the structural information that exists about hexokinase II. This isoenzyme is a 96-kDa monomer [8], about twice as large as hexokinase IV, a 48-kDa monomer [21], and there is evidence [8, 22, 23] that although both are monomers, hexokinase II structurally resembles a dimer of hexokinase IV. It is reasonable to suppose, therefore, that the allosteric site for glucose 6-phosphate evolved from what was originally a second catalytic site [8], a site that must have been capable of binding nucleotide as well as sugar.

We thank Mr Roger Taylor of the muscle biochemistry group in Birmingham for carrying out the two-dimensional electrophoresis of purified hexokinase II, and we are grateful to the Medical Research Council for a grant to I. P. T., A. C.-B. and Professor D. G. Walker.

REFERENCES

1. Colowick, S. P. (1973) in *The Enzymes* (Boyer, P. D., ed.) 3rd edn, vol. 9, pp. 1–48, Academic Press, London and New York.
2. Crane, R. K. & Sols, A. (1954) *J. Biol. Chem. 210*, 597–606.
3. Lazo, P. A., Sols, A. & Wilson, J. E. (1980) *J. Biol. Chem. 255*, 7548–7551.
4. Solheim, L. P. & Fromm, H. J. (1981) *Arch. Biochem. Biophys. 211*, 92–99.
5. Britton, H. G. (1966) *Arch. Biochem. Biophys. 117*, 167–183.
6. Gregoriou, M., Trayer, I. P. & Cornish-Bowden, A. (1981) *Biochemistry, 20*, 499–506.
7. Gregoriou, M., Cornish-Bowden, A. & Trayer, I. P. (1981) *Biochem. Soc. Trans. 9*, 62–63.
8. Holroyde, M. J. & Trayer, I. P. (1976) *FEBS Lett. 62*, 215–219.
9. Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1–5.
10. Britton, H. G. & Dann, L. G. (1978) *Biochem. J. 169*, 29–37.
11. Cornish-Bowden, A. & Gregoriou, M. (1981) *Trends Biochem. Sci. 6*, 149–150.
12. Cha, S. (1968) *J. Biol. Chem. 243*, 820–825.
13. King, E. L. & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1378.
14. Ureta, T., Radojković, J., Lagos, R., Guixé, V. & Núñez, L. (1979) *Arch. Biol. Med. Exp. 12*, 587–604.
15. Pollard-Knight, D., Potter, B. V. L., Cullis, P. M., Lowe, G. & Cornish-Bowden, A. (1982) *Biochem. J. 201*, 421–423.
16. Pollard-Knight, D. & Cornish-Bowden, A. (1982) *Mol. Cell. Biochem. 44*, 71–80.
17. Hanson, T. L. & Fromm, H. J. (1965) *J. Biol. Chem. 240*, 4133–4139.
18. Lueck, J. D. & Fromm, H. J. (1974) *J. Biol. Chem. 249*, 1341–1347.
19. Fromm, H. J. (1975) *Initial Rate Enzyme Kinetics*, pp. 1–21, Springer-Verlag, Berlin, Heidelberg and New York.
20. Ricard, J., Noat, G., Got, C. & Borel, M. (1972) *Eur. J. Biochem. 31*, 14–24.
21. Holroyde, M. J., Allen, M. B., Storer, A. C., Warsy, A. S., Chesher, J. M. E., Trayer, I. P., Cornish-Bowden, A. & Walker, D. G. (1976) *Biochem. J. 153*, 363–373.
22. Cornish-Bowden, A. (1977) *J. Theor. Biol. 65*, 735–742.
23. Darby, M. K. & Trayer, I. P. (1981) *Biochem. Soc. Trans. 9*, 62.
24. Connolly, B. A. & Trayer, I. P. (1979) *Eur. J. Biochem. 93*, 375–385.

M. Gregoriou, Laboratory of Molecular Biophysics, Department of Zoology, University of Oxford,
South Parks Road, Oxford, Great Britain, OX1 3PS

I. P. Trayer and A. Cornish-Bowden, Department of Biochemistry, University of Birmingham,
P.O. Box 363, Birmingham, Great Britain, B15 2TT