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Isotope-Exchange Evidence for an Ordered Mechanism for Rat-Liver Glucokinase, a Monomeric Cooperative Enzyme†

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ABSTRACT: The order of addition of substrates and release of products in the reaction catalyzed by rat-liver glucokinase has been studied by measurements of isotope exchange. Experiments at chemical equilibrium showed some degree of randomness, but steady-state experiments showed a predominantly ordered process with glucose binding first and glucose 6-phosphate released last. Experiments to trap binary complexes in the steady state demonstrated the existence of complexes of the enzyme with glucose and with glucose 6-phosphate but gave no evidence for the occurrence of corresponding complexes with ATP or ADP. Flux ratios measured in both the forward and reverse reactions provided a more rigorous and quantitative confirmation of these characteristics of the reaction. These observations support the interpretation of glucokinase cooperativity in terms of a "mnemonical" mechanism and conflict with an alternative interpretation in terms of a random addition of substrates.

Mammals have evolved diverse mechanisms for controlling the rate of phosphorylation of glucose by ATP,[1] in accordance with the differing metabolic functions of different tissues. In brain and muscle, for example, glucose is phosphorylated to provide for the energetic needs of the tissues, so that the rate ought to depend on the demand for glucose 6-phosphate, not on the supply of glucose: in these tissues, therefore, the predominant isoenzymes of hexokinase are saturated at low glucose concentrations but are highly susceptible to inhibition by glucose 6-phosphate (Weil-Malherbe & Bone, 1951; Crane & Sols, 1954; Hanson & Fromm, 1965; Copley & Fromm, 1967; Bachelard et al., 1971). In the liver, however, the purpose of glucose phosphorylation is not primarily to provide the liver with energy but to regulate the concentration of glucose in the blood, so the rate should depend on the supply of glucose, not on the demand for glucose 6-phosphate. It is not surprising, therefore, that glucokinase (also known as hexokinase type IV), the principal isoenzyme in hepatocytes, is insensitive to glucose 6-phosphate but responds cooperatively to glucose, with a maximum sensitivity to glucose at ~2.5 mM, lower than but comparable with the normal concentration of 5 mM in blood (Niemeyer et al., 1975; Storer & Cornish-Bowden, 1976b). This behavior has been observed not only with the purified enzyme but also with isolated rat hepatocytes and with cell-free extracts (Bontemps et al., 1978).

There is considerable evidence that glucokinase is a monomeric enzyme under all conditions that have been studied, including those that exist under normal assay conditions (Holroyde et al., 1976; Cárdenas et al., 1978, 1979), with only a single binding site for glucose on each molecule (Connolly & Trayer, 1979). This is a highly unusual property for an enzyme with pronounced positive cooperativity and shows that the cooperativity of glucokinase cannot be explained in terms of the quasi-equilibrium models that have dominated ideas about cooperativity for 15 years (Monod et al., 1965; Koshland et al., 1966). Instead, the phenomenon must be purely kinetic in origin.

Storer & Cornish-Bowden (1977) proposed a model for glucokinase based on the "mnemonical" mechanism derived by Ricard et al. (1974) from the concept of "enzyme memory" introduced by Rabin (1967). Rather similar ideas have also been developed by Shill & Neet (1975). In its simplest form the mnemonical mechanism requires glucokinase to exist with two forms of free enzyme that bind glucose with different affinities to give the same enzyme–glucose complex. If this complex is capable of reacting fast enough at high ATP concentrations to prevent equilibration between the two forms of free enzyme and the enzyme–glucose complex, then apparent cooperativity of glucose binding is generated by the changes in the relative proportions of the two forms of free enzyme that occur as the glucose concentration increases. At low ATP concentrations, on the other hand, equilibration of glucose binding cannot be prevented, because the enzyme–glucose complex cannot be removed fast enough; no cooperativity with respect to glucose occurs therefore at very low ATP concentrations. A further postulate of the mechanism is that ATP does not bind to the free enzyme. Thus ATP participates in one step only of the mechanism and therefore no deviation from Michaelis–Menten kinetics with respect to ATP occurs at any glucose concentration. Cárdenas et al. (1979) have interpreted their very similar data for glucokinase in terms of a somewhat more complex model: this resembles the mnemonical model in postulating the existence of distinct forms of free enzyme, but it also includes an alternative order of binding of substrates with ATP capable of binding first. In

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[1] Abbreviations used: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Glc, glucose; G6P, glucose 6-phosphate; enzymes, hexokinase, ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1; glucokinase, also known as hexokinase type IV or type D, ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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principle, a random-order model alone, without any mnemonical characteristics, can account for kinetic cooperativity (Ferdinand, 1966), but Cárdenas et al. (1979) found that such a model could not account in detail for the behavior of glucokinase.

In the present study we have set out to determine whether more than one pathway for reaction exists, and, if so, whether the minor pathway is kinetically significant under ordinary steady-state conditions, and to elucidate the order of binding of substrates and release of products in the major pathway. Although in principle this kind of information is available from product-inhibition studies [see, e.g., Cornish-Bowden (1979)], in practice complex data can be interpreted in various ways that make the approach much more ambiguous than one would wish. We have therefore used measurements of isotope exchange, both at equilibrium and in the steady state, as a way of getting more clear-cut information. Exchange rates at equilibrium provide a highly sensitive indicator of the existence of minor pathways, but do not show whether they contribute appreciably to the reaction rates observed in the steady state. This can only be done by studying the reaction under steady-state conditions, either by experiments to trap binary complexes (Hass & Byrne, 1960) or by measurements of flux ratios (Britton, 1966). We have used both methods in both forward and reverse reactions, measuring all possible exchanges between glucose, glucose 6-phosphate, ATP, and ADP.

Experimental Procedures

Materials. D-[U-^{14}C]glucose (~260 mCi/mmol), adenosine 5′-[γ-^{32}P]triphosphate (triethylammonium salt; ~20 Ci/mmol), [8-^{3}H]adenosine 5′-triphosphate (ammonium salt; 21 Ci/mol), and [U-^{14}C]adenosine 5′-diphosphate (ammonium salt; 500–600 Ci/mol) were purchased from the Radiochemical Centre Ltd. (Amersham, Buckinghamshire, United Kingdom). Adenosine 5′-diphosphate (dimonocyclohexylammonium salt), grade VI, adenosine 5′-triphosphate (disodium salt), D-glucose 6-phosphate (monosodium salt), and imidazole, grade III, were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). All other chemicals were the best grades available and were used as supplied, except for triethylamine, which was purified by distillation to remove a fluorescent contaminant. Yeast hexokinase B (in 1 M NaCl) was a generous gift from Professor E. A. Barnard, Imperial College, London. DEAE-cellulose (DE 52) and DEAE-cellulose paper (DE 81) were purchased from Whatman Ltd. (Maidstone, United Kingdom).

Assay of Glucokinase Activity. Glucokinase was assayed by using the coupled system described by Storer & Cornish-Bowden (1974), with glucose-6-phosphate dehydrogenase as coupling enzyme. The unit of enzyme activity is defined as the amount of enzyme catalyzing the phosphorylation of 1 μmol of glucose/min at pH 8.5, I = 0.15 M, and 30 °C.

Purification of Glucokinase. Glucokinase was purified to homogeneity from the livers of Wistar rats (250–300 g), essentially by the method of Holroyde et al. (1976), with some modifications in accordance with the findings of Wright et al. (1978).

The Stability of Glucokinase under Assay Conditions. Glucose (50 mM) and/or high concentrations of glycerol are essential for the stability of glucokinase (Storer, 1976). In experiments carried out either in the absence of glucose or at glucose concentrations <50 mM, 30% glycerol had to be included in the buffers. At this concentration of glycerol, however, the cooperative behavior of glucokinase with glucose is diminished (unpublished observations). Although this effect is not fully characterized, it was thought unlikely that glycerol would alter the mechanism of addition of substrates and products to the enzyme. As a precaution to check the validity of this supposition, some experiments were carried out in the presence of 1.5 mM DTT, 1 mM EDTA, and 1 mM $MgCl_2$ instead of glycerol. These conditions ensured that the assays were linear for at least the 10 min in which measurements were made but were less effective than 30% glycerol in maintaining enzyme activity, so that no more than 15% was lost in 4 h. This was taken into account in the design of experiments so that it would not impair the results.

Preparation and Purification of Radioactively Labeled 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 by incubation with yeast hexokinase B in the presence of excess unlabeled ATP and glucose, respectively. D-[U-^{14}C]Glucose (3.8 μmol, 260 μCi μmol^{-1}) was freeze-dried and taken up in 0.5 mL of triethylammonium bicarbonate (20 mM, pH 7.5) containing 10 μmol of ATP. Yeast hexokinase B (3.5 units in 10 μL) was added, and the reaction was allowed to proceed overnight at 20 °C. Similarly, adenosine 5′-[γ-^{32}P]triphosphate (0.055 μmol, 18.2 Ci mmol^{-1}) was freeze-dried, taken up in 100 μL of buffer containing 0.15 μmol of glucose and 0.15 μmol of $MgCl_2$, and incubated with 3.5 units of yeast hexokinase overnight at 20 °C. For determination of the extent of reaction, a 1-μL sample was applied to Whatman 1 MM paper and electrophoresed at 3 kV, pH 6.5 (pyridine–acetic acid–water, 25:1:225 v/v/v) for 40 min. This procedure gave a good separation of glucose, ATP, glucose 6-phosphate, and inorganic phosphate. The radioactive compounds were located by scanning a 5-cm strip of the paper containing the sample in a Packard Model 7200 radiochromatogram scanner. Under these conditions >99% of labeled glucose or ATP was converted into product.

Labeled glucose 6-phosphate was purified on a DEAE-cellulose column (10 × 1 cm) equilibrated in 10 mM triethylamine bicarbonate buffer, pH 7.5. (The buffer was prepared by dissolving dry ice in a solution of redistilled triethylammonium in water until it reached pH 7.5 at 20 °C.) The reaction mixture was diluted with distilled water to 1 mL and loaded onto the column, which was subsequently washed with 10 mL of 10 mM triethylammonium bicarbonate buffer and developed with a linear concentration gradient from 10 to 400 mM buffer. The glucose 6-phosphate fractions, electrophoretically homogeneous and free from hexokinase activity, were freeze-dried and redissolved in a small volume of 50 mM imidazole nitrate buffer, pH 6.5, and stored at −20 °C.

Separation of Substrates and Products. Glucose, ATP, ADP, and glucose 6-phosphate can be separated by anion-exchange chromatography on DEAE-cellulose paper using procedures modified from those of Morrison & Cleland (1966) and Sherman (1963). Samples (3 μL) were applied to DEAE-cellulose strips (2.5 × 57 cm) and subsequently developed either (a) by ascending chromatography in 0.6 M ammonium formate buffer, pH 3.1, containing 5 mM EDTA for 5 h at 25 °C or (b) by descending chromatography in distilled water for 4 h at 25 °C. Development in ammonium formate buffer gave a good resolution of all four substrates and products, but elution with distilled water was more effective in separating glucose and glucose 6-phosphate in samples containing high concentrations of either glucose 6-phosphate or glycerol and was used throughout for this separation. Separated reaction components were located by inspection of the chromatograms under ultraviolet light (254 nm) and by scanning the strips in a Packard Model 7200 radiochromatogram scanner.

Measurement of Radioactivity. The amount of radioactivity in substrates and products was determined by liquid scintillation counting in a Phillips PW 4540 liquid scintillation analyzer. The area (~ 6 × 2.5 cm) of the chromatograms containing ^{14}C- and ^{3}H-labeled reactants was cut out, fluted, and counted in 15 mL of toluene scintillation fluid with 80% and 50% efficienty, respectively. For the measurement of ^{32}P, chromatogram pieces were shaken vigorously in vials containing 15 mL of distilled water to give a fine paper suspension, and the radioactivity was determined by Cerenkov counting with 35% efficiency. In all cases radioactivity remained bound to paper and in no case was there any dependence of the counting on paper orientation.

Flux Measurements. In all cases flux measurements were conducted away from equilibrium, in systems consisting either of two substrates and one product or of two products and one substrate. To measure the fluxes from glucose 6-phosphate to ATP and glucose in the presence of either substrate, we set up a reaction mixture containing ADP, glucose 6-phosphate, ATP (or glucose), and both D-[U-^{14}C]glucose 6-phosphate and glucose 6-[^{32}P]phosphate in 25 μL of 50 mM imidazole nitrate buffer, pH 6.5 (or 50 mM glycylglycinate buffer, pH 8.0), containing $MgCl_2$ in 5 mM excess over the total concentrations of ATP and ADP,[2] 100 mM KCl, 1 mM DTT, and either 30% glycerol or an additional 0.5 mM DTT, 1 mM $MgCl_2$, and 1 mM EDTA to maintain enzyme stability. The reaction mixtures, in capped micro test tubes, were equilibrated to 30 °C for 5 min, and reaction was started by adding 6 μL of glucokinase equilibrated in the corresponding buffer. At intervals, pairs of 3-μL samples were removed from the reaction mixture and applied to two DEAE-cellulose strips, and the spots were saturated with absolute ethanol to ensure that the reaction was stopped. These were then developed, one with ammonium formate and the other with distilled water, as indicated above. The rate of conversion of glucose 6-phosphate into glucose and ATP was determined from the slopes of the lines obtained in plots of radioactivity in either glucose or ATP against time.

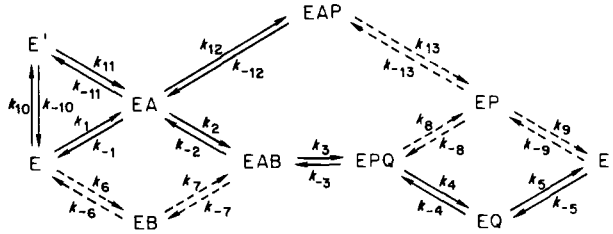
Theory

Isotope Exchange at Equilibrium. Detection of minor pathways in mechanisms by measuring the rates of isotope exchange at chemical equilibrium is a widely used technique, and many accounts of its principles are available [e.g., Fromm (1975)].

Detection of Binary Complexes in Steady State. This method was introduced by Hass & Byrne (1960). It depends on the fact that any exchange from labeled product to substrate can only occur if an enzyme-product complex that is a compulsory reactant in the exchange is present under the conditions of the experiment.

Measurement of Flux Ratios. This method was introduced by Britton (1966) and has been applied to yeast hexokinase (Britton & Clarke, 1972) and rabbit-muscle pyruvate kinase (Dann & Britton, 1978). It is considerably more quantitative and informative than the complex-trapping approach and requires measurement of the rates at which molecules of a product, e.g., Q in Scheme I, are being converted into molecules of two substrates, A and B. These rates are known as

Scheme I: Mnemonical Mechanism with Random Order of Binding of Substrates and Release of Products[a]



[a] In the mechanism postulated for rat-liver glucokinase, A = glucose, B = ATP, P = ADP, and Q = glucose 6-phosphate, and the steps shown by broken lines appear to make a negligible contribution to the reaction under steady-state conditions.

the fluxes from Q to A and from Q to B, and we shall symbolize them as $F(Q \rightarrow A)$ and $F(Q \rightarrow B)$, respectively. They can be measured as the rates of isotope exchange from suitably labeled molecules of Q.

In the context of Scheme I, let us consider the fate of a molecule of Q that binds to the EP complex. The *on* rate of this reaction is simply $k_{-8}[EP][Q]$. Not all of the resulting EPQ molecules react to give EAB, however: some dissociate to EQ + P or EP + Q, and so the fraction that proceed immediately to EAB is only $k_{-3}/(k_{-3} + k_4 + k_8)$. The fraction that reaches EAB eventually is greater than this, however, because a fraction $k_{-4}[P]/(k_{-4}[P] + k_5)$ of the molecules that dissociate to EQ + P is not lost by further dissociation to E + P + Q but instead reassociates (without loss of the original Q molecule) to EPQ. Fractionation of this kind occurs at every step in the reaction, and the expressions for the likelihood that the original Q molecule that bound to EP will eventually emerge as a molecule of A or B are highly complicated. Moreover, the total fluxes from Q to A and from Q to B involve not only Q molecules that begin by binding to EP but also Q molecules that react first with E and then with P. So the expressions for $F(Q \rightarrow A)$ and $F(Q \rightarrow B)$ are even more complicated, and analysis might seem to be a hopeless task. Nonetheless, a large part of the pathways from Q to A is identical with the pathways from Q to B; because of this, some of the terms in the two expressions are the same. Consequently, the expression for the flux ratio $F(Q \rightarrow B)/F(Q \rightarrow A)$ is simpler than the expressions for the individual fluxes and unlike them it is readily amenable to analysis.

The flux ratio is particularly simple if there is a unique pathway for substrate binding, e.g., if EB does not exist so that all of the reaction proceeds through EA.[3] The pathway from Q to A is then identical with that from Q to B apart from the additional step EA → E + A that is required for converting Q to A. The flux ratio is therefore determined by the fraction of EA molecules that release A rather than return to Q. The immediate fate of EA molecules is determined by the rates $v_{-1} = k_{-1}[EA]$ and $v_2 = k_2[EA][B]$. If v_2 is 0 (e.g., if [B] = 0), all EA molecules must release A; hence, $F(Q \rightarrow A) = F(Q \rightarrow B)$ and the flux ratio is unity. But v_2 increases in proportion to [B], and so as [B] increases the proportion of EA molecules that fail to release A increases; hence, the flux ratio increases linearly with [B]. To a first approximation it is given by the expression:

$$\frac{F(Q \rightarrow B)}{F(Q \rightarrow A)} \simeq 1 + v_2/v_{-1} = 1 + k_2[B]/k_{-1}$$

[2] A constant excess of 5 mM $MgCl_2$ over the total concentrations of ATP and ADP is sufficient to ensure that the nucleotides exist in a large and nearly constant proportion as their 1:1 complexes with Mg^{2+} (Storer & Cornish-Bowden, 1976a), which are the forms that undergo reactions catalyzed by glucokinase. This experimental design for controlling ionic concentrations was used in all experiments.

[3] To simplify this discussion, we shall also ignore steps 10, 11, 12, and 13, i.e., those that involve E' and EAP. If these are included, the analysis is more complex, but its essential qualitative features are unaffected (see Appendix).

This is not exact because some of the EAB molecules produced from EA reverse direction again and do eventually yield E + A, and similar fractionation occurs in every step. The correct expression (see Appendix) shows the same kind of dependence on [B], however, and the same lack of dependence on [A] and can be written as

$$\frac{F(Q \rightarrow B)}{F(Q \rightarrow A)} = 1 + \alpha[B]$$

where α is not simply k_2/k_{-1} but is a function of other rate constants and the concentration [P] as well.

If the substrates can bind to the enzyme in random order, with both EA and EB present in significant concentrations in the steady state, fractionation of both of these binary complexes must be taken into account. The same principles apply, however, and so the fractionation of EA still depends on [B] but not on [A], whereas the fractionation of EB depends on [A] but not on [B]. The flux ratio is then given by an expression of the form

$$\frac{F(Q \rightarrow B)}{F(Q \rightarrow A)} = \frac{1 + \alpha[B]}{1 + \beta[B]} \Big/ \frac{1 + \gamma[A]}{1 + \delta[A]}$$

in which α and γ are functions of the rate constants and [P] and β and δ are functions of the rate constants only.

The two kinds of dependence on [A] and [B] given by these expressions for compulsory and random orders of substrate binding are readily distinguishable by experiment, and, when the order is compulsory, they provide unambiguous discrimination between A and B. Moreover, the results are unaffected by various kinds of complication that can make traditional steady-state kinetic analysis almost impossible: since there is no net flux through dead-end reactions, they make no contribution to the expressions for the flux ratios; the order of product release does affect the definitions of α, β, γ, and δ in the above expressions, but it does not affect their forms and thus does not interfere with the discrimination between substrates; any complexities outside the part of the mechanism that connects Q with A and B, such as isomerization of the free enzyme (as postulated in the mnemonical mechanism for glucokinase), have no effect on the flux ratios. These matters are considered more rigorously under Appendix. Because of them it follows that measurements of flux ratios in principle yield much less ambiguous information than the more widely used kinetic techniques.

Results

Isotope Exchange at Equilibrium. The initial rates of the exchanges Glc ⇄ G6P and ATP ⇄ ADP at chemical equilibrium were measured as functions of all four reactant concentrations increased in constant ratio. This approach (Wedler & Boyer, 1972) has the advantage over the more usual method of increasing the concentrations of only one substrate–product pair that it eliminates any competitive effects between the exchanging pair and the pair whose concentrations are raised. Thus, any inhibition of the initial rates of exchange at high reactant concentrations unambiguously indicates an obligatory order of addition of one or both of the reactants involved in the exchange.

The equilibrium constant for the hexokinase reaction was determined by using pure yeast hexokinase B under our experimental conditions. For determination of the equilibrium concentrations of substrates and products, extended time courses of the reaction between glucose, ATP, G6P, and [^{14}C]ADP were followed by measuring the radioactivity in ATP and glucose on paper chromatograms. The equilibrium constant at pH 6.5, expressed in terms of total nucleotide concentrations, was calculated to be [ADP][G6P]/[ATP][Glc] = 234 ± 13, in fair agreement with the value of 386 reported by Robbins & Boyer (1957) for measurements at pH 6.0 under somewhat different conditions.

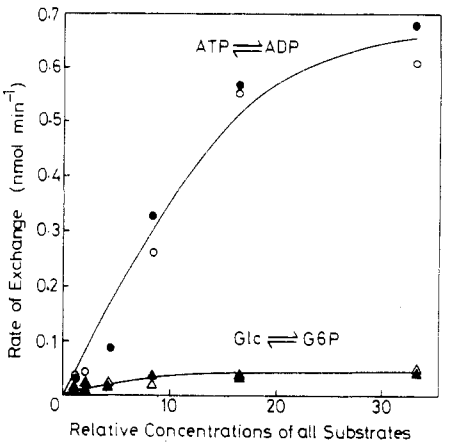


FIGURE 1: Isotope exchange at equilibrium. The initial rates of isotopic exchange for the reactions ATP → ADP (●), ADP → ATP (○), Glc → G6P (▲), and G6P → Glc (△) were measured. The concentrations of all four reactants were raised in constant equilibrium ratio from 0.12 mM ATP, 0.18 mM glucose, 1.4 mM ADP, and 3.6 mM glucose 6-phosphate to 3.9 mM ATP, 6.2 mM glucose, 47 mM ADP, and 121 mM glucose 6-phosphate. Glucokinase (0.9 unit/mL) was incubated with the mixtures of reactants at 30 °C for 90 min, and isotopic exchanges were initiated by addition of the appropriate isotope in separate reactions. Radioactivities were as follows: [8-^{14}C]ATP, 0.83 μCi/mL; [U-^{14}C]glucose, 32 μCi/mL; [U-^{14}C]ADP, 8.3 μCi/mL; [U-^{14}C]glucose 6-phosphate, 320 μCi/mL.

As shown in Figure 1, neither the Glc ⇄ G6P nor the ATP ⇄ ADP exchange was inhibited by raising the reactant concentrations to saturation, suggesting that both substrates and products were capable of binding to the enzyme in random order. The ATP ⇄ ADP exchange was ~19 times faster than the Glc ⇄ G6P exchange. This confirms the occurrence of slowly dissociating enzyme–Glc and enzyme–G6P complexes and suggests that the reaction proceeds preferentially through these complexes. It also demonstrates that the chemical step in the reaction cannot be rate limiting, i.e., glucokinase cannot obey a rapid-equilibrium mechanism. There was no exchange between glucose and glucose 6-phosphate in the absence of ATP or between ATP and ADP in the absence of glucose, confirming that the reaction proceeds by a ternary-complex mechanism. In these last two respects the measurements of isotope exchange provided direct evidence for features of the mechanism that had been suspected from the earlier initial-rate data. Although some degree of randomness was revealed by the measurements at equilibrium (Figure 1), it was not possible to determine how much the minor pathways contributed to the rate in the steady state and thus the extent to which they could be used as an explanation of the kinetic cooperativity of glucokinase.

Detection of Binary Complexes in Steady State. To look for enzyme–G6P and enzyme–ADP complexes, we tested glucokinase as a catalyst for the exchange ADP → ATP in the absence of glucose 6-phosphate and for the exchange G6P → Glc in the absence of ADP (Figure 2). Although ADP exchanges with ATP in the absence of glucose 6-phosphate, there is no exchange between glucose 6-phosphate and glucose in the absence of ADP. This shows that when the reaction is proceeding in the direction of glucose phosphorylation product release occurs via enzyme–G6P and that no enzyme–ADP is detectable.

Similar experiments were done in the reverse direction to look for enzyme–Glc and enzyme–ATP complexes (Figure 3).

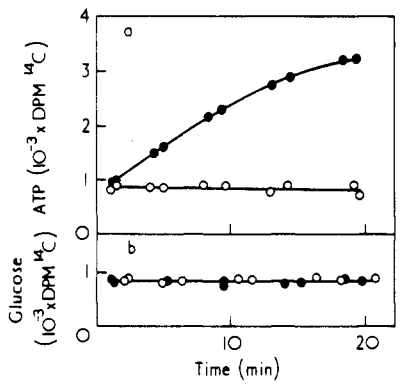


FIGURE 2: Detection of enzyme–product binary complexes in steady state. (a) Flux of ADP to ATP in the absence of glucose 6-phosphate while the chemical reaction proceeds toward production of ADP + glucose 6-phosphate (●). The reaction mixture consisted of 6 mM glucose, 4 mM ATP, and 35 mM [U-^{14}C]ADP (0.2 μCi/μmol) in a final volume of 35 μL of 50 mM glycylglycine buffer, pH 8.0, containing 1 mM DTT, 30% glycerol, 0.1 M KCl, and 5 mM excess $MgCl_2$ over the total ATP + ADP concentration. The reaction was started by adding 0.03 unit of glucokinase. Glucose was omitted from the control (○). (b) Lack of flux from glucose 6-phosphate to glucose in the absence of ADP while the chemical reaction proceeds toward production of ADP + glucose 6-phosphate (●). The reaction mixture was the same as in (a) except that ADP was replaced by 75 mM [U-^{14}C]glucose 6-phosphate (0.38 μCi/μmol). ATP was omitted from the control (○).

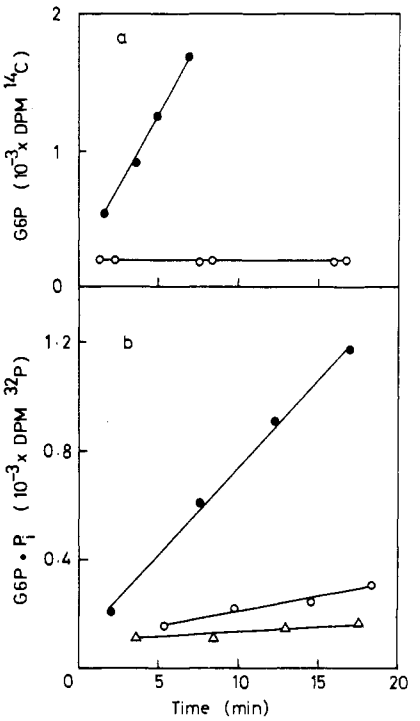


FIGURE 3: Detection of enzyme–substrate binary complexes in steady state. (a) Lack of flux from glucose to glucose 6-phosphate in the absence of ATP while the chemical reaction proceeds toward production of glucose + ATP (○). To eliminate traces of ATP from ADP, we incubated glucokinase at 30 °C overnight with 35 mM ADP and 25 mM glucose. After addition of [U-^{14}C]glucose to a final specific activity of 2.9 μCi/μmol, the reaction was started by adding 15 mM glucose 6-phosphate to a final reaction mixture consisting of 15 mM ADP, 10 mM glucose, and 0.4 unit/mL glucokinase in a total volume of 25 μL of 50 mM imidazole nitrate buffer, pH 6.5. The flux of glucose to glucose 6-phosphate in the presence of 1 mM ATP (●) was measured as a control for enzyme inactivation. (b) Flux from ATP in the absence of glucose while the chemical reaction proceeds toward the production of glucose + ATP. The total flux (●) was measured after adding glucokinase (0.6 unit/mL) to a reaction mixture containing 27 mM glucose 6-phosphate, 15 mM ADP, and 0.75 mM [γ-^{32}P]ATP (48.6 μCi/μmol) in a total volume of 25 μL of 50 mM imidazole buffer, pH 6.5. That this total was predominantly but not totally the flux of interest, i.e., from ATP to glucose 6-phosphate, was shown by setting up reaction mixtures without ADP (○) and without ADP and glucose 6-phosphate (△). The latter small flux was due to the feeble activity of glucokinase as a catalyst for hydrolysis of ATP and the former to this and also to the ability of the small amount of glucose present as a contaminant of glucose 6-phosphate to act as a substrate.

No flux of glucose to glucose 6-phosphate was observed in the absence of ATP, indicating the absence of enzyme–ATP complex under these conditions. However, a large flux from ATP to glucose 6-phosphate was observed in the absence of glucose, confirming the formation of an enzyme–Glc complex.

This last experiment was complicated somewhat by the simultaneous occurrence of two other fluxes that were not distinguished by the assay used: one of these was caused by reaction of labeled ATP with the glucose that occurred as an unavoidable contaminant of the glucose 6-phosphate required at high concentrations in the experiment; the other was due to a feeble catalysis by glucokinase of the hydrolysis of ATP, producing labeled inorganic phosphate, which was not separated from glucose 6-phosphate by the chromatographic system used. As shown in Figure 3, these additional fluxes could be measured independently of the main experiment and did not account for >10% of the total flux observed. Thus, they did not affect the essential conclusion from this experiment that, when the reaction proceeds toward glucose and ATP as products, product release occurs via an enzyme–Glc complex and no enzyme–ATP complex could be detected.

Determination of Flux Ratios. The fluxes G6P → Glc and G6P → ATP were measured by means of glucose 6-phosphate labeled both with ^{14}C and with ^{32}P, and the ratio F(G6P → ATP)/F(G6P → Glc) is plotted in Figure 4 against the concentrations of ATP and glucose. The most reproducible determinations of flux ratios could be made by using substrates of high specific radioactivity and measuring both radioactive products from the same reaction mixture. In this way the flux ratio was not subject to errors arising from any variability of the enzyme between reaction mixtures. The ratio showed a linear increase with the ATP concentration but was independent of the glucose concentration; this agrees exactly with what one would expect if the substrates bound in compulsory order with glucose first and does not permit a random order of addition.

In the converse experiment in which the reverse reaction was studied (Figure 5), the results were similar and again in accord with the experiments to trap binary complexes. The flux ratio F(ATP → ADP)/F(ATP → G6P) increased linearly with the concentration of ADP but was independent of the concentration of glucose 6-phosphate. Again, this rules out a random order of addition of glucose 6-phosphate and ADP but requires a compulsory order with glucose 6-phosphate binding to the free enzyme.

These experiments were done both in the presence and absence of glycerol (which was present in most reaction mixtures to stabilize the enzyme) and led to identical conclusions in both cases. As a further guard against artifactual results on account of different conditions from those used in the earlier initial-rate studies (Storer & Cornish-Bowden, 1977), the experiments to trap binary complexes were carried out at pH 8.0 as well as at pH 6.5; again, there was no significant difference in the results.

Discussion

The classical method of measuring rates of isotope exchange at chemical equilibrium is very sensitive for detecting alternative pathways in enzyme-catalyzed reactions, but it suffers

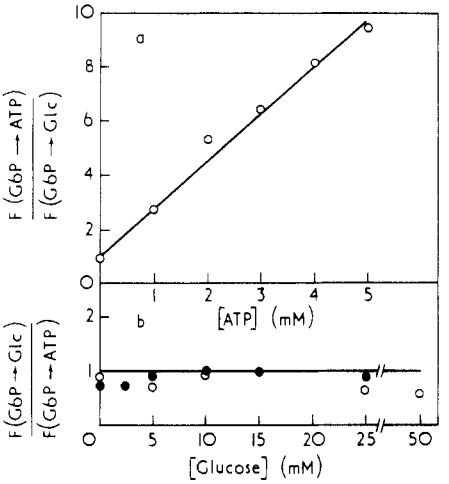


FIGURE 4: (a) Variation of the flux ratio F(G6P → ATP)/F(G6P → Glc) with the concentration of ATP. The ratio was determined from the fluxes of [U-^{14}C]glucose 6-phosphate (4.1 μCi/μmol) to glucose and of glucose 6-[^{32}P]phosphate (1.3 μCi/μmol) to ATP in the presence of 17 mM glucose 6-phosphate, 15 mM ADP, and increasing concentrations of ATP in 50 mM imidazole nitrate buffer, pH 6.5, containing 1.5 mM DTT, 6 mM excess $MgCl_2$, 1 mM EDTA, and 100 mM KCl. (b) Lack of variation of the ratio F(G6P → Glc)/F(G6P → ATP) with the concentration of glucose. The fluxes were measured both in reaction mixtures as in (a) apart from the presence of glucose instead of ATP (●) and also with 1 mM DTT and 30% glycerol instead of 1.5 mM DTT and 1 mM EDTA (○). The horizontal line drawn at a flux ratio of unity is a theoretical line for an ordered addition of substrates.

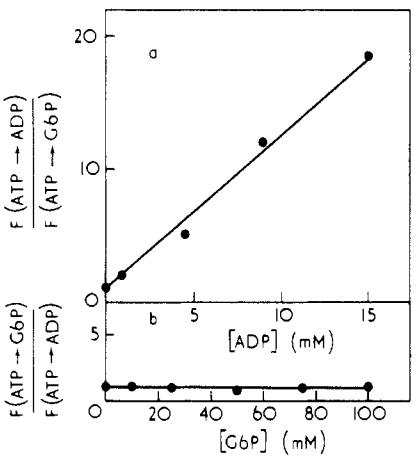


FIGURE 5: (a) Variation of the ratio F(ATP → ADP)/F(ATP → G6P) with the concentration of ADP. The fluxes were measured by using [8-^{3}H]ATP (6.45 μCi/μMol) and [γ-^{32}P]ATP (5.4 μCi/μmol) in the absence of glucose 6-phosphate, in reaction mixtures containing 20 mM glucose, 5 mM ATP, and increasing concentrations of ADP in 50 mM imidazole nitrate buffer, pH 6.5. (b) Lack of variation of the ratio F(ATP → G6P)/F(ATP → ADP) with the concentration of glucose 6-phosphate. Measurements were made as in (a) except that glucose 6-phosphate was present and ADP was absent. The horizontal line drawn at a flux ratio of unity is a theoretical line for an ordered release of products. In both (a) and (b) the rate of ATP hydrolysis was negligible, and therefore no correction of the fluxes was needed.

from the disadvantage that it does not indicate whether a particular pathway is preferred, nor does it discriminate between effects due to an ordered addition of substrates and those due to an ordered release of products (Britton & Dann, 1978). Measurement of flux ratios allows the addition of substrates and the release of products to be studied independently, however, and gives a good indication of the degree of randomness of each. In some cases the two approaches complement one another and both should be used for a realistic characterization of a mechanism. Thus yeast hexokinase was shown by measurements of isotope exchange at equilibrium to follow a random mechanism (Fromm et al., 1964), but Britton & Clarke (1972) subsequently used the flux ratio method to show that is was predominantly ordered, with glucose binding to the free enzyme.

Similarly, in the present study no binary complexes between enzyme and either ATP or ADP could be detected in the steady state, though the existence of an alternative pathway involving both of these complexes seems necessary to account for the failure of the Glc ⇄ G6P exchange at chemical equilibrium to be inhibited by high concentrations of all four reactants (Figure 1). The flux ratio method showed no evidence for such pathways, however, indicating a predominantly ordered reaction in the steady state with glucose binding to the free enzyme and glucose 6-phosphate released last.

Product-inhibition experiments (Storer & Cornish-Bowden, 1977) gave some support to the possibility that glucose 6-phosphate and not ADP might be the first product to be released from the ternary complex. If the ternary complex were highly unstable, so that ATP binding and glucose 6-phosphate release was effectively a single concerted step, as in the "Theorell–Chance mechanism" (Theorell & Chance, 1951), one might expect competitive inhibition between glucose 6-phosphate and ATP, as is observed. This rather implausible interpretation of the product-inhibition data now seems to be definitely excluded by the failure to detect an enzyme-ADP complex and the corresponding success in detecting an enzyme-G6P complex, both in the trapping experiments (Figure 2) and in the measurements of flux ratios (Figure 5). The competitive inhibition between glucose 6-phosphate and ATP must therefore be explained in a different way, perhaps by supposing that they compete for a phosphate binding site on the enzyme.

The measurements of isotope exchange at equilibrium provide some support for the suggestion of Cárdenas et al. (1979) that the glucokinase-catalyzed reaction proceeds in part through an alternative pathway in which ATP binds to the free enzyme. However, no evidence for such a pathway could be detected in the steady state, and so even though it may exist as a minor pathway, it appears to make little contribution to the net reaction. Accordingly, we believe that glucokinase cooperativity is best explained by the mnemonical mechanism proposed previously (Storer & Cornish-Bowden, 1977), with the modification that ADP rather than glucose 6-phosphate must be the first product to be released.

Appendix

Derivation of Flux Expressions. Here we shall derive expressions for the fluxes F(Q → A) and F(Q → B) for the mechanism shown in Scheme I. The objective will not only be to illustrate more rigorously than was done under Theory how flux expressions are obtained and interpreted but also to justify some assertions made there. Scheme I is more general than the mnemonical mechanism previously proposed for glucokinase (Storer & Cornish-Bowden, 1977), since it not only includes the two forms of free enzyme required by that mechanism but also allows substrate binding and product release to occur in random order, as suggested by Cárdenas et al. (1979).

To calculate the flux from Q to A, we must take account of all pathways that accomplish the transformation. We shall denote the flux through any step by the symbol v with the same subscript as the corresponding rate constant k; for example, the flux from E to EQ is

$$F(\mathrm{E} \rightarrow \mathrm{EQ}) = v_{-5} = k_{-5}[\mathrm{E}][\mathrm{Q}]$$

and the fluxes from EQ to EPQ and to E are

$$F(\mathrm{EQ} \rightarrow \mathrm{EPQ}) = v_{-4} = k_{-4}[\mathrm{EQ}][\mathrm{P}]$$

$$F(\mathrm{EQ} \rightarrow \mathrm{E}) = v_5 = k_5[\mathrm{EQ}]$$

The flux through two consecutive steps, such as $F(\mathrm{E} \rightarrow \mathrm{EQ} \rightarrow \mathrm{EPQ})$, is given by the flux through the first step multiplied by the fraction of molecules of intermediates that undergo the second step: in this case the flux through the first step is v_{-5}, and the fraction of molecules that undergo the second step is $v_{-4}/(v_{-4} + v_5)$, so

$$F(\mathrm{E} \rightarrow \mathrm{EQ} \rightarrow \mathrm{EPQ}) = v_{-4}v_{-5}/(v_{-4} + v_5)$$

This flux accounts for only part of the flux from Q to EPQ, however, because addition of Q to EP occurs simultaneously with flux v_{-8}. Since these two fluxes occur in parallel, the total flux is the sum of the two, i.e.

$$\begin{aligned} F(\mathrm{Q} \rightarrow \mathrm{EPQ}) &= F(\mathrm{EP} \rightarrow \mathrm{EPQ}) + F(\mathrm{E} \rightarrow \mathrm{EQ} \rightarrow \mathrm{EPQ}) \\ &= v_{-8} + v_{-4}v_{-5}/(v_{-4} + v_5) \end{aligned}$$

Similarly, the reverse flux is

$$F(\mathrm{EPQ} \rightarrow \mathrm{Q}) = v_8 + v_4v_5/(v_{-4} + v_5)$$

We can now continue to write down the flux from Q to EAB and its reverse in a similar manner:

$$\begin{aligned} F(\mathrm{Q} \rightarrow \mathrm{EAB}) &= \frac{F(\mathrm{Q} \rightarrow \mathrm{EPQ})F(\mathrm{EPQ} \rightarrow \mathrm{EAB})}{F(\mathrm{EPQ} \rightarrow \mathrm{EAB}) + F(\mathrm{EPQ} \rightarrow \mathrm{Q})} \\ &= \frac{v_{-3}[v_{-8} + v_{-4}v_{-5}/(v_{-4} + v_5)]}{v_{-3} + v_8 + v_4v_5/(v_{-4} + v_5)} \end{aligned}$$

$$\begin{aligned} F(\mathrm{EAB} \rightarrow \mathrm{Q}) &= \frac{F(\mathrm{EAB} \rightarrow \mathrm{EPQ})F(\mathrm{EPQ} \rightarrow \mathrm{Q})}{F(\mathrm{EPQ} \rightarrow \mathrm{Q}) + F(\mathrm{EPQ} \rightarrow \mathrm{EAB})} \\ &= \frac{v_3[v_8 + v_4v_5/(v_{-4} + v_5)]}{v_{-3} + v_8 + v_4v_5/(v_{-4} + v_5)} \end{aligned}$$

and the flux from EAB to A is obtained in the same way:

$$F(\mathrm{EAB} \rightarrow \mathrm{A}) = v_{-7} + \frac{v_{-2}[v_{-1} + v_{-11} + v_{12}v_{13}/(v_{-12} + v_{13})]}{v_2 + v_{-1} + v_{-11} + v_{12}v_{13}/(v_{-12} + v_{13})}$$

These last three expressions can now be assembled into an expression for $F(\mathrm{Q} \rightarrow \mathrm{A})$:

$$F(\mathrm{Q} \rightarrow \mathrm{A}) = \frac{F(\mathrm{Q} \rightarrow \mathrm{EAB})F(\mathrm{EAB} \rightarrow \mathrm{A})}{F(\mathrm{EAB} \rightarrow \mathrm{A}) + F(\mathrm{EAB} \rightarrow \mathrm{Q})}$$

and an expression for $F(\mathrm{Q} \rightarrow \mathrm{B})$ can be obtained in an exactly similar way.

The flux expressions are too complicated to be amenable to analysis as they stand but they can be made so by dividing one by the other to give an expression for the flux ratio. The main advantage of this division is that when the flux ratio is rewritten in terms of rate constants, all concentrations of intermediates cancel, whereas they do not cancel from the individual flux expressions. The dependence of the ratio on the substrate concentrations can be written in a rather simple way

$$\frac{F(\mathrm{Q} \rightarrow \mathrm{B})}{F(\mathrm{Q} \rightarrow \mathrm{A})} = \frac{1 + \alpha[\mathrm{B}]}{1 + \beta[\mathrm{B}]} \Big/ \frac{1 + \gamma[\mathrm{A}]}{1 + \delta[\mathrm{A}]}$$

where

$$\alpha = \frac{k_2(k_{-7} + f[\mathrm{P}])}{\{k_{-1} + k_{-11} + k_{12}k_{13}[\mathrm{P}]/(k_{-12} + k_{13})\}(k_{-2} + k_{-7} + f[\mathrm{P}])}$$

$$\beta = \frac{k_2k_{-7}}{\{k_{-1} + k_{-11} + k_{12}k_{13}[\mathrm{P}]/(k_{-12} + k_{13})\}(k_{-2} + k_{-7})}$$

$$\gamma = \frac{(k_{-2} + f[\mathrm{P}])k_7}{(k_{-2} + k_{-7} + f[\mathrm{P}])k_{-6}}$$

$$\delta = \frac{k_{-2}k_7}{(k_{-2} + k_{-7})k_{-6}}$$

$$f[\mathrm{P}] = \frac{k_3\{k_8 + k_4k_5/(k_{-4}[\mathrm{P}] + k_5)\}}{k_{-3} + k_8 + k_4k_5/(k_{-4}[\mathrm{P}] + k_5)}$$

It follows that for this mechanism a plot of the flux ratio against either [A] or [B] is a rectangular hyperbola with a limiting value of unity at vanishing concentrations of both substrates.

If the substrates bind to the enzyme in a compulsory order, e.g., A first followed by B, the expression for the flux ratio is greatly simplified. We can then put $k_{-6} = k_6 = k_{-7} = k_7 = 0$, in which case $\beta = \gamma = \delta = 0$, only α remaining finite, so

$$\frac{F(\mathrm{Q} \rightarrow \mathrm{B})}{F(\mathrm{Q} \rightarrow \mathrm{A})} = 1 + \alpha[\mathrm{B}]$$

Thus, in this case the flux ratio is a linear function of [B] and is independent of [A]; more generally, it is a linear function of the concentration of the second substrate to bind to the enzyme and independent of that of the first.

These expressions are similar to those derived by Britton (1966) for an enzyme obeying hyperbolic kinetics. The differences in some of the coefficients result from the fact that Scheme I allows for differential binding of A to two forms of the free enzyme and it includes the nonproductive complex EAP. Any other discrepancies between our equations and those of Britton & Dann (1978) are caused by typographical errors in the subscripts of some rate constants in the latter.

A major advantage of the flux ratio approach is that the results are qualitatively unaffected by mechanistic complexities that can make conventional steady-state analysis very difficult. For example, the existence of two forms of free enzyme with different properties has no qualitative effect on the analysis: this may be seen by putting $k_{-11} = 0$ in the above expressions, a change that leaves them unchanged in form. Second, as there is no flux through a dead-end step, such steps are irrelevant to the flux ratio. Although steps 12 and 13 in Scheme I are not dead-end steps and they do affect the flux ratio, their effect is quantitative rather than qualitative, and they do not obscure the essential discrimination between a random and a compulsory order of binding. Finally, the effect of assuming a random order of product release may be seen by assuming that only EQ exists, i.e., that $k_{-8} = k_8 = k_{-9} = k_9 = k_{-13} = k_{13} = 0$; in this case the form of the dependence of the flux ratio on [P] is altered but the form of its dependence on [A] and [B] is not; again the discrimination between two pathways of substrate binding in unaffected by considerations of the order of product release.

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Irreversible Inhibition of Glutamate Decarboxylase by α-(Fluoromethyl)glutamic Acid†

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ABSTRACT: α-(Fluoromethyl)glutamic acid (FMG) was synthesized and shown to be an active site directed irreversible inhibitor of glutamate decarboxylase (EC 4.1.1.15) from *Escherichia coli*. The K_I for the active enantiomer is 1.4 μM, and the $k_{inh} = 5.9 \times 10^{-3}$ s^{-1}. Substrates for the enzyme, such as L-glutamate, and competitive inhibitors, such as citrate, decrease the rates of FMG-mediated inactivation of the enzyme. A profound change in the ultraviolet spectrum of the enzyme accompanies the inactivation process. When [^{3}H]-FMG is used, it can be shown that the enzyme incorporates radioactivity at the same rate as that of inactivation. There is a 1:1 stoichiometry of [^{3}H]FMG incorporated to pyridoxal phosphate binding subunits of the enzyme. From these and other studies it is concluded that FMG is a substrate for the enzyme and alkylates it as a consequence of this turnover.

Glutamate decarboxylase is a pyridoxal phosphate linked enzyme which catalyzes the decarboxylation of L-glutamate to γ-aminobutyric acid (Strausbauch & Fischer, 1967). Several active site directed irreversible inhibitors of this enzyme, including bromopyruvate, have been reported (Fonda, 1976). Reagents of this type, however, generally lack potency and selectivity. Recently, studies from different laboratories have shown that α-fluoromethyl substrate analogues can be potent mechanism-based inactivators of pyridoxal phosphate linked decarboxylases. For example, α-fluoromethyl-Dopa and α-(difluoromethyl)ornithine are potent inactivators of Dopa decarboxylase and ornithine decarboxylase, respectively (Maycock et al., 1980; Metcalf et al., 1978). Since we have been interested in the design of inactivators of glutamate decarboxylase, we have synthesized several of the fluorinated glutamate analogues as possible mechanism-based inactivators of this enzyme. In this report, we demonstrate that α-(fluoromethyl)glutamate is a potent mechanism-based inactivator of bacterial glutamate decarboxylase. The K_I for the inhibitor is 1.4 μM, and the first-order rate constant for inactivation

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