Kinetic Evidence for a 'Mnemonic' Mechanism for Rat Liver Glucokinase

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Inhibition studies of glucokinase were carried out with the products of the reaction, glucose 6-phosphate and MgADP^- , as well as with ADP^{3-} , Mg^{2+} and ATP^{4-} . The results of these, together with those of kinetic studies of the uninhibited reaction described previously [Storer & Cornish-Bowden (1976) *Biochem. J.* **159**, 7–14], indicate that the enzyme obeys a 'mnemonic' mechanism. This implies that the co-operativity observed with glucose as substrate arises because glucose binds differentially to two forms of the free enzyme that are not in equilibrium under steady-state conditions. The mechanism predicts the decrease in glucose co-operativity observed at low concentrations of MgATP^{2-} . The product-inhibition results suggest that glucose 6-phosphate is released first and that it is possibly displaced by MgATP^{2-} in a concerted reaction.

Rat liver glucokinase (ATP-D-glucose 6-phosphotransferase, EC 2.7.1.2) displays a co-operative dependence of the rate of reaction on the glucose concentration, with a degree of co-operativity that increases with the MgATP^{2-} concentration (Storer & Cornish-Bowden, 1976b). However, no co-operativity can be detected for the dependence of the rate on the MgATP^{2-} concentration. This is difficult to reconcile with a simple model in which the steady-state rate is assumed to mimic the binding of substrates at equilibrium (Monod *et al.*, 1965; Kirtley & Koshland, 1967), because such models usually require that a ligand that alters the homotropic interactions of another ligand should itself display homotropic interactions. This does not rule out the agency of a quasi-equilibrium model more complex than those considered by previous authors, but it does suggest that steady-state models, such as those proposed by Ferdinand (1966) and Rabin (1967), ought to be considered for glucokinase.

Glucokinase appears to be monomeric under all conditions, with a mol.wt. of about 48 000 (Holroyde *et al.*, 1976), approximately one-half the molecular weights of the mammalian hexokinases (Colowick, 1973), which are also monomeric and are similar in amino acid composition both to one another and to glucokinase; a new method of analysis (Cornish-Bowden, 1977a) of the data for glucokinase and for hexokinase type II (Holroyde & Trayer, 1976) indicates about 85% identity between the primary sequences of the two enzymes. These observations not only exclude models based on reversible association of glucokinase (Frieden, 1967; Nichol *et al.*, 1967), but they also raise difficulties for all quasi-equilibrium explanations of glucokinase co-operativity. It would be necessary to postulate the presence of at least two interacting glucose-binding sites on the

same polypeptide chain for glucokinase, and presumably four or more similar sites on each polypeptide chain for the hexokinases. This seems unlikely [though not unprecedented, as Kretsinger (1972) has found multiple calcium-binding sites on the single polypeptide chain of the calcium-binding protein from carp muscle], and again suggests that steady-state models should be considered.

Although a number of steady-state models of co-operativity have been proposed (Ferdinand, 1966; Rabin, 1967; Griffin & Brand, 1968; Sweeny & Fisher, 1968), and indeed any mechanism that includes branched pathways can in principle account for complex kinetics (Wong & Hanes, 1962), there is little experimental evidence for such models. One enzyme that resembles glucokinase and appears to require a steady-state explanation of its complex kinetics is hexokinase type L_1 from wheat-germ. Meunier *et al.* (1974) have shown that its properties are consistent with a 'mnemonic' model derived by Ricard *et al.* (1974) from the concept of 'enzyme memory' introduced by Rabin (1967) and developed by Whitehead (1970). The essential feature of this concept is that the enzyme relaxes relatively slowly from one state to another and 'remembers' its interaction with a ligand for a perceptible period after the ligand has been released or transformed into another species. This permits homotropic interactions between two binding processes that are separated by time rather than space, a phenomenon that is impossible for binding at equilibrium because equilibria are independent of time.

The experiments described previously (Storer & Cornish-Bowden, 1976b) did not provide sufficient evidence to permit the choice of a single model as the simplest explanation of glucokinase co-operativity. We have now studied the inhibition of the enzyme by products and substrate analogues, with a view to decreasing the range of possible explanations.

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Materials and Methods

Materials

ADP and glucose 6-phosphate (both sodium salts) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. They were converted before use into their tetramethylammonium salts by the method given previously for ATP and NADP⁺ (Storer & Cornish-Bowden, 1976b). The concentration of ADP in stock solutions was determined by the spectrophotometric method of Bock *et al.* (1956) and that of glucose 6-phosphate by the ferricyanide assay for total reducing sugar (Park & Johnson, 1949). The glucose content of glucose 6-phosphate solutions was measured with a glucose assay kit (Blood Sugar GOD-Perid method) from Boehringer, Mannheim, Germany, and was repeatedly found to be 0.2–0.5% of the total reducing sugar present. Although this degree of contamination may seem trivial, it implies that 50 mM-glucose 6-phosphate (for example) would contain 0.1–0.25 mM-glucose, sufficient to cause errors if ignored in experiments with less than 10 mM-glucose. Any glucose added with the glucose 6-phosphate was therefore allowed for in calculating and stating glucose concentrations in the present paper. Because of the slow but significant spontaneous hydrolysis of glucose 6-phosphate at pH 8, fresh solutions were prepared each day. Effects of progressive deterioration of these solutions were minimized by using glucose concentrations in a convoluted sequence as described previously (Storer & Cornish-Bowden, 1976b) and by measuring the glucose contamination at the midpoint of each experiment.

Other materials were as given previously (Storer & Cornish-Bowden, 1976b).

Concentrations of complex ions

Concentrations of the various ionic species in the reaction mixtures were calculated as described by Storer & Cornish-Bowden (1976a).

For solutions that contain both ATP and ADP, 1 mM excess of MgCl₂ over the total concentrations of ATP and ADP is not enough to ensure that ADP exists in a high and nearly constant proportion as MgADP[−]. Inhibition experiments with MgADP[−] were done therefore with a 5 mM excess of MgCl₂, which provided a good compromise between the need to maintain high proportions of both MgATP^{2−} (88–90%) and MgADP[−] (76–82%) and the need to minimize inhibition by Mg²⁺.

At high concentrations (50 mM) glucose 6-phosphate interferes with the use of MgCl₂ excess to control the concentration of MgATP^{2−}, because most of the Mg²⁺ excess is sequestered as a complex with glucose 6-phosphate. In studies of inhibition by glucose 6-phosphate, therefore, the concentration of

MgATP^{2−} was controlled by keeping the total ATP concentration in a 1 mM excess over the total MgCl₂ concentration; this had an effect similar to that of the usual excess of MgCl₂ over ATP, but was less affected by competing anions. (It is less suitable for routine use in the absence of glucose 6-phosphate, however, because glucokinase is more sensitive to ATP^{4−} than it is to Mg²⁺.) With a 1 mM excess of ATP over MgCl₂, and in the presence of 50 mM-glucose 6-phosphate, the concentration of MgATP^{2−} was maintained in the range 78–87% of the total MgCl₂ concentration (0.1–10 mM), and the glucose 6-phosphate existed almost entirely as its dianion (96.5–98%).

The ionic strength (*I*) was kept as close as possible to 0.15 by the addition of tetramethylammonium chloride. This value was chosen because the concentrations of all of the species of interest exhibit stationary points at *I* 0.15 and vary very little in the range *I* 0.1–0.2 (Storer & Cornish-Bowden, 1976a).

In studies of inhibition by species other than Mg²⁺ the concentration of Mg²⁺ was kept as small and as constant as possible, the actual values being calculated as described previously (Storer & Cornish-Bowden, 1976a). In the glucose 6-phosphate experiments the Mg²⁺ concentration was in the range 0.008–0.16 mM; in the MgADP[−] experiments it was in the range 3.5–4.2 mM; in the ADP^{3−} experiments it was in the range 0.04–0.15 mM; and in the ATP^{4−} experiments it was in the range 0.04–0.05 mM. In the MgADP[−] experiments the concentrations of Mg²⁺ were high enough in comparison with the inhibition constant (7 mM) to cause perceptible inhibition, but the variation in this inhibition was much too small to account for the effects attributed to MgADP[−]. In the other experiments the inhibition attributable to Mg²⁺ was negligible.

For calculating the concentrations of complexes the thermodynamic data cited previously (Storer & Cornish-Bowden, 1976a) were used, together with data of Phillips *et al.* (1966) and Blair (1970) for ADP complexes and data of Hammes & Kochavi (1962) for glucose 6-phosphate.

Enzyme assays

The coupled assay with glucose 6-phosphate dehydrogenase as coupling enzyme (Storer & Cornish-Bowden, 1976b) was used in all experiments except those in which inhibition by glucose 6-phosphate was being studied. In these the rate was measured by coupling the production of ADP to the oxidation of NADH by means of pyruvate kinase and lactate dehydrogenase as coupling enzymes. The need for excess of ATP in these experiments (see above) introduced problems with the assay, because pyruvate kinase requires free Mg²⁺ (MacFarlane & Ainsworth, 1972) and is inhibited

by MgATP^{2-} and ATP^{4-} . To overcome these effects and to ensure valid assays [see Storer & Cornish-Bowden (1974) for criteria], it was necessary to use higher concentrations of both coupling enzymes than those given previously (Storer & Cornish-Bowden, 1976b); 20 units each of pyruvate kinase and lactate dehydrogenase were used in each assay at the highest MgATP^{2-} concentrations, and up to 40 units per assay at the lowest MgATP^{2-} concentrations (when the concentration of free Mg^{2+} was at its lowest).

Inhibition constants

The inhibition constants K_i and K_i' are defined empirically in the present paper, according to the following equation:

$$v = \frac{Vs}{K_m \left(1 + \frac{i}{K_i}\right) + s \left(1 + \frac{i}{K_i'}\right)} \quad (1)$$

which expresses the initial rate v in terms of s , the variable substrate concentration, i , the inhibitor concentration, and four constants V , K_m , K_i and K_i' . Inhibition has a competitive component if K_i is finite and the inhibitor decreases the apparent value of V/K_m ; it has an uncompetitive component if K_i' is finite and the inhibitor decreases the apparent value of V . These definitions are not necessarily equivalent to the mechanistic definitions preferred by some workers (e.g. Thompson & Bachelard, 1976). K_i values were estimated by the method of Dixon (1953), K_i' values by the method of Cornish-Bowden (1974).

Derivation of rate equations

Steady-state rate equations were derived with the aid of the computer program described in the preceding paper (Cornish-Bowden, 1977b). In cases where the kinetic behaviour could be predicted by

inspection, any predictions made were checked by comparison with the results from the computer program.

Curve-fitting

In all cases recorder traces were essentially straight during the periods of measurement and initial rates were found with a ruler and pencil. These were fitted to theoretical equations by the non-linear regression procedure of Cornish-Bowden & Koshland (1970), modified as described by Wharton *et al.* (1974). Each observed rate, v , was assigned a weight $\hat{v}^{-2}$, where $\hat{v}$ was the best-fit rate, because trial experiments (Storer *et al.*, 1975) had shown that such weights approximated to the correct weights better than any of the alternatives that were tried. Programs were run on an International Computers Ltd. 1906A computer.

Results

Inhibition studies

The results of inhibition studies with glucose 6-phosphate, MgADP^- , ADP^{3-} , Mg^{2+} and ATP^{4-} are summarized in Table 1. As a guide to the extent and precision of the data, Fig. 1 shows primary plots for inhibition by glucose 6-phosphate with MgATP^{2-} as variable substrate, plotted as described by Dixon (1953) and by Cornish-Bowden (1974). The plots show that the inhibition was largely or entirely competitive, with $K_i = 11.5 \text{ mM}$ and K_i' very large. Inhibition by glucose 6-phosphate was more complex when glucose was the variable substrate (Fig. 2), because of deviations from the Michaelis-Menten equation at low glucose concentrations (Storer & Cornish-Bowden, 1976b), but at glucose concentrations greater than 10 mM the inhibition was mixed, with $K_i \approx K_i' \approx 60 \text{ mM}$.

Table 1. *Inhibition constants for glucokinase*

The inhibition constants K_i and K_i' are defined empirically as given in the Materials and Methods section, and refer to conditions in which the Michaelis-Menten equation is obeyed by both substrates, i.e. glucose concentrations greater than 10 mM.

Inhibitor	[Glucose] (mM)	[MgATP^{2-}] (mM)	Type of inhibition	K_i (mM)	K_i' (mM)
Glucose 6-phosphate	Varied	4.3	Mixed	60	60
	50.0	Varied	Competitive	11.5	High
MgADP^-	Varied	4.4	Mixed	10–15	2.1
	50.0	Varied	Mixed*	1.4	4.0
ADP^{3-}	Varied	4.4	Mixed	8–10	8–10
	50.0	Varied	Mixed	2.3	>20
Mg^{2+}	Varied	4.1	Mixed	7.0	>50
	50.0	Varied	Mixed	7.5	>50
ATP^{4-}	Varied	0.96	Mixed	>25	4.1
	50.0	Varied	Competitive	2.4	High

* Curved primary plots (see the text and Fig. 3).

Fig. 1. Inhibition of glucokinase by glucose 6-phosphate at various MgATP^{2-} concentrations

Initial rates, v , were measured at 30°C in 50 mM-tetramethylammonium glycyglycinate buffer, pH 8.0, by the coupled assay with pyruvate kinase and lactate dehydrogenase as coupling enzymes as described under 'Enzyme assays'. The concentration of glucose was 50 mM. (a) The competitive component of the inhibition was investigated by plotting $1/v$ against inhibitor concentration (Dixon, 1953). Concentrations of MgATP^{2-} were 8.8 mM (○), 2.58 mM (●), 1.72 mM (△), 0.645 mM (▲) and 0.344 mM (□). (b) The uncompetitive component of the inhibition was investigated by plotting $[\text{MgATP}^{2-}]/v$ against inhibitor concentration (Cornish-Bowden, 1974). The parallel lines indicate that this component is not detectable, i.e. K'_i approaches infinity. Concentrations of MgATP^{2-} were 2.58 mM (○), 1.72 mM (●), 0.86 mM (△), 0.645 mM (▲) and 0.344 mM (□).

Apart from MgADP^- , the other inhibitors listed in Table 1 gave primary plots qualitatively similar to those for glucose 6-phosphate. MgADP^- behaved in

a more complicated fashion (Fig. 3), with curved primary plots; for this inhibitor the inhibition constants given in Table 1 were obtained from the extrapolated curves and thus refer to the behaviour at low MgADP^- concentration.

Effect of inhibitors on glucose co-operativity

At high MgATP^{2-} concentrations MgADP^- had no effect on the glucose co-operativity, but at low MgATP^{2-} concentrations the slight co-operativity was decreased by MgADP^- and was not seen at 4.3 mM- MgADP^- (Fig. 4). The highest Hill coefficient (h) seen in these experiments was 1.64, rather higher than the highest value (1.47) seen in the experiments without any inhibitor (Storer & Cornish-Bowden, 1976b), but this difference was probably no more than a reflection of the different preparations of enzyme and of the difficulty of estimating reproducibly the maximum slope of a curve; it was not a consequence of the higher concentration of free Mg^{2+} required for MgADP^- -inhibition experiments, because a separate experiment showed the co-operativity to be independent of Mg^{2+} .

Glucose 6-phosphate had a negligible effect on the glucose co-operativity. A preliminary experiment suggested otherwise, as the Hill coefficient seemed to decrease from 1.67 to 1.43 as the glucose 6-phosphate concentration was increased from 0 to 20 mM. However, this proved to be an artifact of the glucose present in the stock solution of glucose 6-phosphate: when this glucose was allowed for as described in the Materials and Methods section, the effect was almost abolished.

Discussion

Model-fitting

In the light of these inhibition studies and the molecular-weight studies described elsewhere (Holroyde *et al.*, 1976), we have fitted our results for the uninhibited reaction (Storer & Cornish-Bowden, 1976b) to various models, of which the one shown in Scheme 1 seems to be the simplest reasonable model consistent with the observations. (To avoid unnecessary complication we shall defer discussion of alternative models until later in the present paper.) Scheme 1 represents a compulsory-order ternary-complex 'mnemonic' mechanism similar to the one used by Meunier *et al.* (1974) to explain their observations with wheat-germ hexokinase type L_1 , but with the inclusion of a non-productive complex in which glucose and MgADP^- are bound simultaneously to the enzyme. This complex provides the simplest explanation of the curved primary plots observed for inhibition by MgADP^- (Fig. 3), and, more important, logic demands that, if the enzyme

Fig. 2. Inhibition of glucokinase by glucose 6-phosphate at various glucose concentrations

Conditions were the same as in Fig. 1, apart from substrate concentrations, which were as follows: the concentration of $MgATP^{2-}$ was 4.3 mM, the concentrations of glucose were 50 mM (○), 30 mM (●), 20 mM (△), 10 mM (▲) and 5 mM (□). (a) Plot of $1/v$ against inhibitor concentration; (b) plot of $[glucose]/v$ against inhibitor concentration.

is capacious enough for glucose and $MgATP^{2-}$ to be bound simultaneously, it must also be capacious enough for glucose and $MgADP^-$ to be bound simultaneously. Thus omission of a non-productive ternary complex would be more in need of explanation than inclusion of one.

In the absence of products, the initial rate, v , is given for Scheme 1 by an equation of the following form:

$$v = \frac{(1 + \beta_1[glucose])\beta_2[glucose][MgATP^{2-}]}{1 + \beta_3[MgATP^{2-}] + (1 + \beta_4[MgATP^{2-}])\beta_5[glucose] + (1 + \beta_6[MgATP^{2-}])\beta_7[glucose]^2} \quad (2)$$

in which there are seven constants to be estimated, from β_1 to β_7 . The results given previously (Storer & Cornish-Bowden, 1976b) provide the best-fit parameters listed in Table 2 when fitted to this equation. One can judge the adequacy of the equation by using the duplicate observations to partition the residual

sum of squares into lack-of-fit and pure-error components as described by Draper & Smith (1966). The results of this analysis of variance (Table 3) show that the lack of fit is on the borderline of significance at the 95% level of confidence, but the non-linearity of eqn. (2) makes an exact analysis of variance impossible, and the significance can only be calculated approximately.

Effects of $MgATP^{2-}$ and products on glucose cooperativity

One of the most satisfying aspects of the model shown in Scheme 1 is the straightforward way in which it accounts for the effects of $MgATP^{2-}$ on glucokinase. Not only is it clear from inspection that

Fig. 3. Inhibition of glucokinase by MgADP^- at variable MgATP^{2-} concentrations

Initial rates, v , were measured at 30°C in 50 mM-tetramethylammonium glycylglycinate buffer, pH 8.0, by the coupled assay with glucose 6-phosphate dehydrogenase as coupling enzyme as described previously (Storer & Cornish-Bowden, 1976b). The concentration of glucose was 50 mM, and concentrations of MgATP^{2-} were 8.8 mM (○), 1.76 mM (●), 0.88 mM (△), 0.44 mM (▲), 0.176 mM (□) and 0.088 mM (■).

the Michaelis-Menten equation should be obeyed for MgATP^{2-} but not for glucose, but it is also easy to see why glucose co-operativity should increase with the MgATP^{2-} concentration. If this concentration is lowered sufficiently, the binding of MgATP^{2-} to the enzyme-glucose complex must become rate-limiting. When this is so, the two forms of the free enzyme must be in equilibrium with one another and with the enzyme-glucose complex, so at very low MgATP^{2-} concentrations the laws of equilibria should apply and there should be no co-operativity in glucose binding. But at high MgATP^{2-} concentrations the conversion of enzyme-glucose complex into products can be fast enough to prevent equilibration of glucose binding, so the laws of equilibria need not apply, and co-operativity is possible.

These considerations apply equally well to the mnemonic model used by Meunier *et al.* (1974) to

Fig. 4. Dependence of glucose co-operativity on MgADP^- concentration

The Hill coefficient (h) was measured as described by Storer & Cornish-Bowden (1976b), at various concentrations of MgADP^- . Results are shown for experiments with 4.4 mM- MgATP^{2-} (●) and 0.44 mM- MgATP^{2-} (○).

Scheme 1. Mnemonic model for glucokinase

account for the kinetics of hexokinase type L_1 from wheat-germ. It is puzzling, therefore, that the (negative) co-operativity observed for glucose with that enzyme was independent of the MgATP^{2-} concentration over a wide range. Further, the same

authors found that their model predicted that co-operativity should be independent of the rate of conversion of the enzyme-substrate complex into products (Ricard *et al.*, 1974); in other words, substrate binding need not be at equilibrium, even if the net reaction is vanishingly slow. The likely reason for this discrepancy between predictions is that Ricard *et al.* (1974) defined co-operativity in terms of a parameter, Γ , that measures the curvature of a double-reciprocal plot at the $1/v$ axis, i.e. at infinite substrate concentration. Unlike the Hill coefficient, h , Γ is independent of the rate of conversion of the enzyme-substrate complex into products. For several reasons we believe h to be preferable to Γ as an index of co-operativity: not only is it widely used already, but it can be estimated without extrapolation; it is not derived from the double-reciprocal plot; and it is more clearly related to the phenomenon of interest, i.e. interaction between binding processes. It would be interesting to know whether h varies with $[\text{MgATP}^{2-}]$ for the data of Meunier *et al.* (1974), but this cannot, unfortunately, be determined with any certainty from the published values of Γ .

The complete rate equation for Scheme 1, including product terms, is complicated, and rigorous analysis of

the effects of products on the Hill coefficient is difficult. However, the model does appear to give a reasonable explanation of the observed effects. Neither of the two product-release steps on the main reaction pathway can affect the relative rates of glucose release and MgATP^{2-} binding, and so they should not affect glucose co-operativity. But, at low MgATP^{2-} concentrations, MgADP^- can prevent the rapid conversion of the enzyme-glucose complex into products by sequestering it as non-productive ternary complex, thereby decreasing the co-operativity; at high concentrations MgATP^{2-} competes effectively with MgADP^- and thus prevents this effect.

Product inhibition

The curvature of the primary plots for inhibition by MgADP^- is explained in terms of Scheme 1 by the fact that MgADP^- acts both as a product inhibitor and as an MgATP^{2-} analogue. (The term 'dead-end inhibitor' is inappropriate here because, according to Scheme 1, the non-productive complex can release glucose to give the same enzyme- MgADP^- complex that appears on the main reaction pathway. If this step, which is mechanistically reasonable, were omitted, the rate equation would contain no terms in $[\text{MgADP}^-]^2$ and there would be no explanation of the curved primary plots.) The curvature is apparent only at low MgATP^{2-} concentrations (Fig. 3) because at high concentrations MgATP^{2-} competes effectively for the enzyme-glucose complex.

Since the inhibition results in Table 1 refer to conditions where the Michaelis-Menten equation is obeyed for both substrates (i.e. at high glucose concentrations), they are most simply rationalized by ignoring the distinction between the two forms of the free enzyme, which is important only when the Michaelis-Menten equation is not obeyed. Then the model simplifies to an ordinary compulsory-order ternary-complex mechanism in which glucose is the first substrate to bind and glucose 6-phosphate is the first product to be released. The expected inhibition patterns for this type of mechanism are well known (see, e.g., Cornish-Bowden, 1976); glucose 6-

Table 2. *Best-fit parameters for glucokinase*

The data for the uninhibited reaction (Storer & Cornish-Bowden, 1976b) were fitted to eqn. (2). Each b_i value in the Table is the least-squares estimate of the corresponding β_i value in eqn. (2). The standard errors are calculated with 145 degrees of freedom (i.e. 152 observations minus seven parameters).

Parameter	Units	Value	Standard error	
			(actual)	(%)
b_1	mM^{-1}	1.24	0.13	(10.6)
b_2	$\text{mM}^{-1} \cdot \text{min}^{-1}$	0.0214	0.0013	(5.9)
b_3	mM^{-1}	6.85	0.51	(7.4)
b_4	mM^{-1}	1.46	0.21	(14.3)
b_5	mM^{-1}	0.80	0.12	(14.5)
b_6	mM^{-1}	2.237	0.0087	(3.9)
b_7	mM^{-2}	0.141	0.015	(10.9)

Table 3. *Comparison between lack of fit and pure error*

The residual sum of squares is defined as the sum of terms of the form $(v - \hat{v})^2/\hat{v}^2$, summed over 152 observations, where v is the observed rate and $\hat{v}$ is the best-fit rate calculated from eqn. (2) with the parameter values listed in Table 2. The Table is constructed as described by Draper & Smith (1966). The observed value of the variance ratio, 1.59, is equal to the upper 5% percentage point of the F -distribution with 106 and 39 degrees of freedom. However, the significance is only approximate because eqn. (2) is non-linear.

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Residual	145	0.43803		
Lack of fit	106	0.35567	0.00336	1.59
Pure error	39	0.08236	0.00211	

phosphate should be a mixed inhibitor whichever substrate concentration is varied, and MgADP^- should be a competitive inhibitor with respect to glucose and a mixed inhibitor with respect to MgATP^{2-} . The non-productive ternary complex introduces terms in $[\text{glucose}][\text{MgADP}^-]$ into the rate equation, however, and thus converts the predicted competitive inhibition by MgADP^- with respect to glucose into mixed inhibition. These predictions are in accordance with the observations if the competitive inhibition by glucose 6-phosphate is regarded as a limiting case of mixed inhibition in which the uncompetitive component is too small to be detected. Although MgADP^- is indeed a mixed inhibitor with respect to both substrates, it is predominantly uncompetitive with respect to glucose and predominantly competitive with respect to MgATP^{2-} ; this implies that non-productive binding of MgADP^- to the enzyme-glucose complex is the more important cause of MgADP^- inhibition.

Dead-end inhibition

Table 1 also includes results for studies of dead-end inhibition by ADP^{3-} , Mg^{2+} and ATP^{4-} . ADP^{3-} is a mixed inhibitor with respect to both substrates, but the competitive and uncompetitive components are equal (pure non-competitive inhibition) when glucose is the variable substrate, whereas the competitive component predominates when MgATP^{2-} is the variable substrate. This is consistent with the reasonable view that ADP^{3-} acts as a MgADP^- analogue, i.e. that ADP^{3-} and glucose bind simultaneously without interaction, whereas ADP^{3-} and MgATP^{2-} cannot bind simultaneously. ATP^{4-} appears to behave as a MgATP^{2-} analogue, again reasonably, as it appears to form a dead-end ternary complex by binding to the enzyme-glucose complex; inhibition by ATP^{4-} is competitive with respect to MgATP^{2-} but largely uncompetitive with respect to glucose. The inhibition by Mg^{2+} is more puzzling, as it is predominantly competitive with respect to both substrates, with the same K_i value. This is reasonable for MgATP^{2-} , but it is not obvious why Mg^{2+} should affect the binding of a non-ionic species such as glucose, except perhaps by inducing an unfavourable conformational change.

Other models

The data for the uninhibited reaction can be fitted adequately to models other than that shown in Scheme 1. For example, if glucokinase had two interacting active sites, or if it followed a random-order ternary-complex mechanism in which the binding of substrates was not maintained at equilibrium (Ferdinand, 1966), the rate equation would resemble eqn. (2) but with additional terms in $[\text{MgATP}^{2-}]^2$. If these

terms were negligible in the experimental range of MgATP^{2-} concentrations, the kinetic behaviour would be indistinguishable on the basis of initial-velocity measurements from that predicted by the mnemonical model. The predictions for product inhibition would be more complicated, but they could be reconciled with the observed behaviour. However, the mnemonical model is more attractive than these alternatives, for several reasons. In view of the absence of any perceptible curvature in the primary plots for MgATP^{2-} (Storer & Cornish-Bowden, 1976b) it would be artificial to argue the presence of undetected terms in $[\text{MgATP}^{2-}]^2$ in the rate equation. Moreover, the mnemonical model is simpler than the alternatives, and it accounts very neatly for the decrease in glucose co-operativity at low MgATP^{2-} concentrations.

There is no doubt that Scheme 1 could be made more complicated without greatly altering its predictions, and this might help to make it more plausible. For example, it is surprising that glucose 6-phosphate should be released before MgADP^- , as it resembles glucose more closely than it resembles MgATP^{2-} . Moreover, kinetic studies of the hexokinases have indicated that they release MgADP^- first or that they release MgADP^- and glucose 6-phosphate in random order (for reviews see Colowick, 1973; Purich *et al.*, 1973). The considerable structural similarities between glucokinase and the hexokinases (Holroyde *et al.*, 1976) make it unlikely that there are major differences in mechanism. It would be difficult to rule out a random release of products from glucokinase, but the evidence suggests that the order shown in Scheme 1 is kinetically the more important. In particular, the competitive inhibition between glucose 6-phosphate and MgATP^{2-} implies either that both compete for the free enzyme, which is ruled out by the fact that MgATP^{2-} should bind after glucose if the co-operativity is to be explained, or that MgATP^{2-} displaces glucose 6-phosphate from the enzyme-glucose complex, as shown in Scheme 1. Strictly this would predict mixed inhibition, but it would tend towards competitive if the displacement were largely concerted, i.e. if the ternary complex with glucose and MgATP^{2-} bound simultaneously were too unstable to exist for an appreciable period. But, if glucose 6-phosphate were released after MgADP^- it could not be a competitive inhibitor with respect to the second substrate, MgATP^{2-} .

The inhibition by glucose 6-phosphate with respect to MgATP^{2-} would be truly competitive if MgATP^{2-} displaced glucose 6-phosphate from the enzyme in a fully concerted reaction, so that the ternary complex occurred only as a transition state, because all terms in $[\text{MgATP}^{2-}][\text{glucose 6-phosphate}]$ would disappear from the rate equation. Concerted displacement reactions were often proposed or assumed in the

earliest studies of two-substrate reactions (Doudoroff *et al.*, 1947; Theorell & Chance, 1951; Koshland, 1954), but it is now usually assumed that ternary complexes should occur as intermediates rather than as transition states (Cleland, 1970). If such a proposal were adopted for glucokinase, it would be logical to suppose that the conversion of the enzyme-glucose complex into the enzyme-MgADP⁻ complex should also be a concerted reaction, as it is closely analogous to the displacement of glucose 6-phosphate by MgATP²⁻. This change would not affect the predictions of Scheme 1, because we have assumed that binding of glucose to the enzyme-MgADP⁻ complex is insignificant.

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