Kinetics of Rat Liver Glucokinase

CO-OPERATIVE INTERACTIONS WITH GLUCOSE AT PHYSIOLOGICALLY SIGNIFICANT CONCENTRATIONS

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The kinetics of glucokinase from rat liver were studied over wide ranges of glucose and MgATP\(^{2-}\) concentrations. The initial rate shows a co-operative dependence on the glucose concentration, with Hill coefficients in the range 1.2–1.5. The degree of glucose co-operativity increases with the MgATP\(^{2-}\) concentration, but no co-operativity was detected for the dependence of the rate on the MgATP\(^{2-}\) concentration. The effects observed occur at physiologically reasonable concentrations of glucose and MgATP\(^{2-}\) and are consistent with the presumed function of glucokinase in maintaining a constant concentration of glucose in the blood.

It has long been recognized that a major function of the mammalian liver is to regulate the concentration of glucose in the blood, and that this is achieved by synthesis and storage of glycogen when the blood-glucose concentration rises, and by breakdown of glycogen when it falls (Bernard, 1848, 1855). In accordance with the usual principles of feedback control (Stadtman, 1970), one might expect the conversion of glucose into glycogen to be controlled at the first step, i.e. the phosphorylation of glucose by ATP. So the enzyme responsible for this reaction ought to be sensitive to the glucose concentration. In fact, the isoenzymes of hexokinase (ATP-\(\beta\)-hexose 6-phosphotransferase, EC 2.7.1.1) found in most tissues, i.e. hexokinase types I, II and III, are barely affected by changes in the blood-glucose concentration, because they are all largely saturated throughout the physiological range (Hanson & Fromm, 1965; Copley & Fromm, 1967; Bachelard et al., 1971), and they are strongly and allosterically inhibited by glucose 6-phosphate at low concentrations. This means that they are well adapted to supply tissues with glucose at a rate that depends on the need (indicated by the concentration of glucose 6-phosphate) and not significantly on the supply. By the same token, however, they are poorly adapted to regulate the supply.

The observation that liver slices can phosphorylate glucose at a rate that varies with the glucose concentration (Cahill et al., 1958) suggested that a hexokinase with a much higher Michaelis constant should exist in the liver, and led to the discovery of glucokinase (ATP-\(\beta\)-glucose 6-phosphotransferase, EC 2.7.1.2, sometimes also referred to as hexokinase type IV) (Walker, 1962; DiPietro et al., 1962; Viñuela et al., 1963), which is half-saturated in the physiological range of glucose concentrations. Further, it is inhibited only weakly and non-allosterically by glucose 6-phosphate (Viñuela et al., 1963; Parry & Walker, 1966), so the ability of the liver to phosphorylate glucose at high concentrations of glucose 6-phosphate is readily explainable. Glucokinase has a greater specificity for glucose than the low-\(K_m\) hexokinases, though the specificity is incomplete (Parry & Walker, 1966).

To shed light on the physiological role of glucokinase, and as a first step in elucidating its mechanism of action, we have studied the kinetics of glucokinase from rat liver over a wide range of concentrations of glucose and MgATP\(^{2-}\). The results are reported in this paper.

Materials and Methods

Glucokinase

Glucokinase was purified from the livers of well-fed Wistar rats by the method described by Holroyde et al. (1976), with the use of materials as given there. For most of the kinetic studies the last stages of the purification (ion exchange on DEAE-Sephadex and gel filtration on Sephadex G-200) were omitted, and the enzyme used had a specific activity of about 20 units/mg of protein rather than the 150 units/mg that was obtained for the homogeneous enzyme. Tests for contaminating enzymes

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showed that no hexokinase, glucose dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucone isomerase, phosphomannose isomerase, glucose 6-phosphatase, adenylate kinase or adenosine triphosphatase activity was present in the preparation. Glucose 6-phosphate dehydrogenase was detected, but there was no point in removing it as it was a constituent of the coupled assay normally used (see below). Comparison between samples of the homogeneous and partially purified preparations of glucokinase revealed no differences in initial-rate behaviour, apart from the difference in specific activity. The partially purified enzyme was preferred because it could be obtained more quickly and in higher yield, and it lost activity more slowly when stored, and far more slowly under assay conditions.

After purification, glucokinase was stored in 0.3M-KCl, 50 mm-glucose, 0.5 mm-dithiothreitol and 20% glycerol at pH 7.2 and 0–2°C. Under these conditions it was usable for several months, losing activity at a rate of about 5% per month. The composition of the storage mixture was established by thorough testing and all of the components contributed to the stability. Several other possible stabilizing materials were tested, of which some, such as ascorbate, accelerated inactivation, and others, such as MgATP2− (instead of glucose) and 10 mm-mercaptoethanol, were less effective than the components used. The high concentration of glucose in the storage mixture necessitated special precautions in some of the kinetic experiments. When very low concentrations were required the excess was removed by absorbing the enzyme on DEAE-cellulose and eluting it with glucose-free storage mixture. Residual glucose was determined with a glucose assay kit (blood sugar GOD–Perid method) from Boehringer, Mannheim, W. Germany. Glucose added with the enzyme was always allowed for when calculating the glucose concentrations in reaction mixtures. (Neglect of this correction would decrease or eliminate the apparent sigmoidicity of the kinetics and might give a spurious suggestion of hexokinase contamination.) The glucose in the storage mixture was not the cause of the kinetic behaviour described below, as glucokinase that had been stored in the absence of glucose behaved in the same way.

Other materials

ATP (disodium salt) was obtained from Kyowa Hakko Kogyo Co., Tokyo, Japan. Dithiothreitol, NADH, NADP+, phosphoenolpyruvate, glucose 6-phosphate dehydrogenase (type VII, from baker’s yeast), pyruvate kinase (type III, from rabbit muscle), lactate dehydrogenase (type XI, from rabbit muscle), and Dowex 50 (X8) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tetramethylammonium hydroxide, tetramethylammonium chloride, Zerolit DM-F and 1M stock solutions of MgCl2 were obtained from BDH Chemicals, Poole, Dorset, U.K. All other reagents were AnalR or best quality reagent grade from Fisons Scientific Apparatus, Loughborough, Leics., U.K., or from BDH Chemicals.

Solutions of reagents were made up in glass-distilled water and the pH was adjusted when necessary by adding 1 M-HCl or 1 M-KOH.

Preparation of standard solutions

ATP and NADP+ were obtained as sodium salts, but were converted into tetramethylammonium salts before use. Dowex 50 (X8) was washed with 1 M-NaOH, 1 M-HCl and glass-distilled water, and was then converted into its tetramethylammonium form by washing with an excess of tetramethylammonium hydroxide, followed by water until the pH of the wash was neutral. Solutions of the sodium salts of ATP or NADP+ were adjusted to pH 7.0 and then stirred gently with the calculated equivalent of tetramethylammonium Dowex. The solution was filtered and the solute concentration determined, ATP by the spectrophotometric method of Bock et al. (1956), and NADP+ by a similar method (ε260 = 15000 litre·mol−1·cm−1). Contamination of ATP with ADP was determined by coupling the conversion of ADP into ATP to the oxidation of NADH, by using pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate (see under ‘Enzyme assays’). ADP was found to account for 1–2% of the total nucleotide content of fresh solutions, and increased only slightly after the frozen solution was stored for 2 weeks.

Glucose 6-phosphate dehydrogenase was obtained as a suspension in 3.2 M-(NH4)2SO4 and was dialysed overnight against 20 mm-Tris/HCl buffer at pH 7.5 before use. The activity after dialysis was about double that of the undialysed enzyme, because of relief of inhibition by sulphate. Pyruvate kinase and lactate dehydrogenase were obtained as salt-free freeze-dried powders and were dissolved in 20 mm-Tris/HCl at pH 7.5 before use.

MgATP2− concentrations

The concentrations of MgATP2− in reaction mixtures were calculated from the total concentrations of Mg2+ and ATP as described by Storer & Cornish-Bowden (1976), by using the thermodynamic data of Phillips et al. (1966). In all reaction mixtures, the total Mg2+ concentration exceeded the total ATP concentration by 1.0 mM. This ensured that the MgATP2− concentration would be a high and nearly constant proportion (86±2%) of the total ATP concentration, that the ATP4− concentration would be small and proportional to the MgATP2− concentration, and that the Mg2+
concentration would be small (about 0.4 mM) and nearly constant (Storer & Cornish-Bowden, 1976).

**Enzyme assays**

One unit of enzyme activity is defined as the amount of enzyme required to catalyse the conversion of 1 µmol of substrate in 1 min at 30°C, in the complete assay mixture. The method used in most of the kinetic experiments was a coupled assay based on that of DiPietro & Weinhouse (1960), with glucose 6-phosphate dehydrogenase as coupling enzyme, under the detailed conditions (apart from glucose and MgATP$^{2-}$ concentrations) given previously (Storer & Cornish-Bowden, 1974). The reaction was followed by measuring the change in $E_{340}$ in a Gilford recording spectrophotometer, model 2400-S, with digital print-out.

Another coupled assay was also used, in which the formation of ADP was coupled to the oxidation of NADH (0.25 mM) by addition of pyruvate kinase (10 units), lactate dehydrogenase (10 units) and excess of phosphoenolpyruvate (1 mM). The reaction was carried out at 30°C in a total volume of 0.75 ml of tetramethylammonium glycyglycinate buffer (50 mM), pH 8.0, containing 100 mM-KCl, 0.25 mM-NADH, glucose, ATP and MgCl$_2$ (1 mM excess over ATP). The mixture was preincubated for 10 min at 30°C before addition of glucokinase, to convert any ADP present into ATP.

The phosphorylation of glucose by ATP is accompanied by the production of an additional ionizable group. The reaction can therefore be followed in a pH-stat (cf. Gutfreund & Hammond, 1963), and this was done in some experiments, primarily to check that effects observed with the other assays were not artifacts of the coupled enzyme systems. The equipment used was obtained from Radiometer, Copenhagen, Denmark, and consisted of a TTT2 titrator with SBR3 titrigraph, ABU12 autoburette and TTA31 titration assembly. The pH was maintained at 8.0 by addition of 10 mM-KOH and the reaction mixture contained 100 mM-KCl, glucose, ATP and MgCl$_2$ (1 mM excess over ATP), in a total volume of 2.0 ml. All solutions were made up in glass-distilled water that had been treated with Zerolit DM-F to remove dissolved CO$_2$, and the reaction was carried out under a stream of CO$_2$-free N$_2$.

With all three assays, whenever the concentration of an ion was varied during a series of experiments, the ionic strength was kept constant by addition of tetramethylammonium chloride.

**Analysis of results**

Initial rates were obtained from the recorder traces with a ruler and pencil. No correction for curvature was necessary because the traces were essentially straight during the first 3 min of each assay.

To minimize any effects due to enzyme inactivation during a series of experiments, substrate concentrations were varied in a convoluted sequence, e.g. if concentrations in the range 2.5–100 mM were required a sequence such as 100, 50, 20, 10, 2.5, 5, 15, 25, 75 mM would be used. Duplicates (when measured) were obtained by repeating the sequence. In the event, no enzyme inactivation during a series of experiments was detected.

Kinetic constants given in this paper were obtained by eye from plots of $s/v$ against $s$, where $s$ is the concentration of the varied substrate and $v$ is the measured rate. In all cases the experimental error was small enough for non-linear regression analysis (cf. Wharton et al., 1974) to make no appreciable difference to the results.

**Results**

**Dependence of the rate on the glucose concentration**

The glucokinase-catalysed rate of glucose phosphorylation, $v$, was measured at several fixed MgATP$^{2-}$ concentrations, with the glucose concentration varied in the range 0.1–100 mM. For glucose concentrations above 10 mM (Fig. 1), the Michaelis–Menten equation was obeyed accurately, with a Michaelis constant for glucose of 5 mM, independent of the MgATP$^{2-}$ concentration. But at lower glucose concentrations plots of [glucose]$/v$ against [glucose] were strongly curved (Fig. 2). The curvature agrees with the deviations from Michaelis–Menten kinetics observed previously (Parry & Walker, 1967; González et al., 1967; Niemeyer et al., 1975), but is more striking because results are shown at lower glucose concentrations, so that more of the curve is experimentally attested. Moreover, the form of plot used here gives a clearer indication of the behaviour at low concentrations than the double-reciprocal plot used by earlier workers. Niemeyer et al. (1975) comment that their results are different in character from those of Parry & Walker (1967), because they consider it unreasonable to draw pairs of straight lines through their experimental points. Actually this difference is more apparent than real, and results partly from the different ranges of glucose concentrations used in the two laboratories and partly from the subjective nature of lines drawn through experimental points (see Cornish-Bowden & Koshland, 1975). If the experimental points of Parry & Walker (1967) and Niemeyer et al. (1975) are compared, with the lines omitted and over the same range of glucose concentrations (2–50 mM), the differences are slight.

The curvature in Fig. 2 indicates a sigmoid or co-operative dependence of the velocity on the glucose concentration, as may be seen more clearly by plotting $v$ against [glucose] (Fig. 6, below). The
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The results shown in Figs. 1–3 were obtained with the coupled assay with glucose 6-phosphate dehydrogenase as coupling enzyme. To rule out any possibility that the deviations from simple kinetics were an artifact of this assay, the experiments were repeated on a more limited scale by using either the pH-stat assay or the coupled assay with pyruvate kinase and lactate dehydrogenase as coupling enzymes. These assays gave similar results, with the same sigmoid dependence of the rate on the glucose concentration.

**Dependence of the rate on the MgATP$^{2-}$ concentration**

When the rate was measured as a function of the [MgATP$^{2-}$]→0 and an upper limit of $h\rightarrow1.47$ as [MgATP$^{2-}$]→∞.

The sigmoidicity can be expressed by the maximum slope $h$ of a Hill plot (of log[v/(V_{app}− v)]) against log[glucose], where $V_{app}$ is the apparent maximum velocity at each concentration of MgATP$^{2-}$, and varies with the MgATP$^{2-}$ concentration as shown in Fig. 3. The curve has the appearance of a rectangular hyperbola through the point (0,1) (though there is presumably no reason why it should be accurately hyperbolic) and a plot of [MgATP$^{2-}$]/(h−1) against [MgATP$^{2-}$] is nearly straight (Fig. 3, inset). This secondary plot indicates a lower limit of $h\rightarrow1.0$ as

[Fig. 1. Dependence of the glucokinase-catalysed rate on the glucose concentration at high glucose concentrations](#)

Initial rates, $v$, were measured at 30°C in 50 mM tetramethylammonium glycylglycinate buffer at pH 8.0, containing 1 mM-NADP$^+$, 100 mM-KCl, ATP (total concentrations in the range 0.125–5 mM), MgCl$_2$ (total concentrations 1 mM in excess of total ATP concentrations), glucose (10–100 mM, as indicated), glucokinase (0.077 unit/ml) and glucose 6-phosphate dehydrogenase (0.8 unit/ml). Concentrations of MgATP$^{2-}$ were calculated (Storer & Cornish-Bowden, 1976) to be 4.3 mM (○), 2.15 mM (●), 0.86 mM (▲), 0.43 mM (●), 0.215 mM (□) and 0.108 mM (■).

[Fig. 2. Dependence of the glucokinase-catalysed rate on the glucose concentration at low glucose concentrations](#)

Initial rates were measured at glucose concentrations in the range 0.1–25 mM, as indicated. Other conditions were as for Fig. 1.
MgATP$_2^-$ concentration, at several fixed glucose concentrations, Michaelis–Menten kinetics were observed in all cases (Fig. 4). Secondary plots (Fig. 5) were in agreement with the results expected from the variable-glucose experiments, i.e. plots of both primary slopes and primary intercepts against [glucose] were linear at high glucose concentrations (above 10 mM), but the plot of slopes curved upwards at low glucose concentrations. Just as the Michaelis constant for glucose at high glucose concentrations was independent of the MgATP$_2^-$ concentration (Fig. 1), so also does the Michaelis constant for MgATP$_2^-$ approach a glucose-independent value of 0.55 mM at high glucose concentrations.

Discussion

The results show that the slight deviations from Michaelis–Menten kinetics observed by earlier workers (Parry & Walker, 1967; González et al., 1967; Niemeyer et al., 1975) are real and substantial, so that plots of rate against glucose concentration are sigmoid. The Hill coefficient ranges from 1.0 at low MgATP$_2^-$ concentrations to about 1.5 at high MgATP$_2^-$ concentrations. The smallness of this upper limit, by comparison with values observed for other co-operative enzymes, helps to explain why the deviations from simple kinetics observed previously were so slight. The suggestion of Parry & Walker (1967) that their results might be explained by a mixture of enzyme forms is untenable, because any deviations due to such a cause would be in the opposite sense (Childs & Bardsley, 1975).

Under conditions where the Michaelis–Menten equation is obeyed, the Michaelis constant for glucose is 5 mM, appreciably lower than the values of 10–20 mM reported by earlier workers (Salas et al., 1965; Parry & Walker, 1967; Pilks, 1972; Grossman et al., 1974). The discrepancy can be explained by the facts that previous results were analysed as double-reciprocal plots, which are liable to frustrate any attempts to obtain realistic estimates of kinetic constants (see Eisenthal & Cornish-Bowden, 1975).
and the curvature of the 'linear' plots was overlooked or its importance was underestimated. Our results actually agree closely with those of Pilks (1972), despite his reported value of 12.5 mM for the Michaelis constant for glucose: the line shown on his plot actually defines a value of 8 mM, and, if the manifest curvature of the plot is allowed for, a value of 4-5 mM is reasonable for the limit at high glucose concentrations.

The sigmoidicity of plots of rate against glucose concentration is greatest at high MgATP$^{2-}$ concentrations (Fig. 3), but it is perceptible over the likely physiological range, and its disappearance at very low MgATP$^{2-}$ concentrations is unlikely therefore to be of physiological consequence. The effect of the sigmoidicity is that glucokinase is most sensitive to the glucose concentration at about 2.5 mM-glucose, i.e., at a physiologically reasonable value, rather than at the origin, where a high sensitivity would be of no account. The value of $dv/d[glucose]$ is about 140% higher at 2.5 mM-glucose than the corresponding value for an enzyme that obeys the Michaelis-Menten equation with $K_m = 5$ mM and gives the same rate at 2.5 mM-glucose (Fig. 6). This advantage is somewhat less if the comparison is made with an enzyme with a very high $K_m$ value, but glucokinase is 60% more sensitive than the most sensitive possible Michaelis-Menten enzyme and uses a far higher proportion of the catalytic potential at concentrations in the physiological range. Ideally one might expect the maximum sensitivity to occur at 5 mM-glucose rather than 2.5 mM. But this discrepancy is fairly small and in view of uncertainties about the detailed conditions in the cell it would be imprudent to conclude much from it.

Fig. 5. Dependence of apparent Michaelis-Menten parameters on the glucose concentration

The intercept on the ordinate ($K_{m,app.}/V_{app.}$) and slope ($1/V_{app.}$) were estimated for each of the plots of [MgATP$^{2-}$]/$v$ against [MgATP$^{2-}$] shown in Fig. 4. (a) Plot of $K_{m,app.}[glucose]/V_{app.}$ against [glucose]; (b) plot of [glucose]/$V_{app.}$ against [glucose].

![Graph of Fig. 5](image)

Fig. 6. Sigmoid dependence of rate on glucose concentration

The observed dependence (-----) of the rate on the glucose concentration at 4.3 mM-MgATP$^{2-}$ is compared with the dependence (-----) expected for an enzyme that obeys the Michaelis-Menten equation with $K_m = 5$ mM and gives the same rate at 2.5 mM-glucose.

![Graph of Fig. 6](image)
Fig. 7. Effect of glucose and MgATP$^2^-$ on cycling between glucose and glucose 6-phosphate in the liver

The Figure shows a hypothetical dependence of the net flux from glucose to glucose 6-phosphate for a cycling system in which the rate of glucose phosphorylation varies with the glucose concentration according to the Michaelis–Menten equation and the rate of glucose 6-phosphate hydrolysis is constant. The three curves represent different MgATP$^2^-$ concentrations and the broken line is drawn for zero flux.

Start (1973) and by Hue & Hers (1974) of cycling in the liver between glucose and glucose 6-phosphate. The two main effects not taken into account in those analyses were the dependence of the rate on the MgATP$^2^-$ concentration and the sigmoidicity of its dependence on the glucose concentration. It is simplest to consider these separately, i.e. to discuss first the effect of [MgATP$^2^-$] dependence as if glucokinase were not co-operative. The point of zero flux, at which the glucokinase and glucose 6-phosphatase reactions are exactly balanced, would then be highly dependent on the MgATP$^2^-$ concentration (Fig. 7) so that, at lower MgATP$^2^-$ concentrations, higher glucose concentrations would be required to achieve net flux towards glycogen. But if the main function of the cycling in the liver between glucose and glucose 6-phosphate is to regulate the concentration of glucose in the blood, a strong dependence on the MgATP$^2^-$ concentration would be unhelpful, and perhaps damaging, even in the rather narrow range (1.4–2.1 mmol/kg wet wt.) that has been reported for the ATP content of the liver (Greenbaum et al., 1971).

This analysis suggests that a possible function of co-operativity in glucokinase is to decrease the effect of MgATP$^2^-$ on the cycle between glucose and glucose 6-phosphate. If the curves in Fig. 7 at higher MgATP$^2^-$ concentration are made sigmoid, without altering their shapes at high glucose concentration, but the curves at lower MgATP$^2^-$ concentration are changed less, the net effect is to bring the points of zero flux closer together, so that the direction of flux is made more dependent on the glucose concentration and less dependent on the MgATP$^2^-$ concentration.

Although eventually it would be desirable to express these conclusions in more precise numerical terms, it would be unprofitable at the present state of knowledge, because not enough is known about the properties of glucose 6-phosphatase (see Arion et al., 1975) or about the local concentrations of glucose, MgATP$^2^-$ and glucose 6-phosphate, or indeed other metabolites that may affect the system. For the present, therefore, it is sufficient to conclude that the properties of glucokinase described in this paper are qualitatively in accordance with its presumed role in maintaining a constant concentration of glucose in the blood (Walker, 1966).

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References

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