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Kinetics of hexokinase D ('glucokinase') with inosine triphosphate as phosphate donor

Loss of kinetic co-operativity with respect to glucose

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When ATP, the normal phosphate donor for hexokinase D ('glucokinase'), is replaced by ITP, the positive co-operativity with respect to glucose disappears. This may be rationalized in relation to kinetic models for hexokinase D co-operativity, which assume that with the normal substrates the chemical reaction and subsequent release of products occur so rapidly that binding of substrates cannot approach equilibrium and is therefore not constrained by the thermodynamic requirement that the Hill coefficient for substrate binding cannot exceed the number of binding sites. ITP is a much poorer substrate than ATP, however: its K_m value at high glucose concentrations is 24 times the value for ATP, whereas the value of the limiting rate V is decreased about 8-fold. Consequently it is no longer possible for the ternary complex to be converted into products rapidly enough to generate kinetic co-operativity. The negative co-operativity with respect to glucose observed in ^2H_2O with ATP as phosphate donor also disappears when ITP is used instead of ATP.

INTRODUCTION

Hexokinase D, which is also known as hexokinase type IV and has in the past often been called 'glucokinase', is the isoenzyme of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) mainly responsible for the phosphorylation of glucose in the vertebrate liver. Although a monomeric enzyme with only a single binding site for glucose (Holroyde *et al.*, 1976; Connolly & Trayer, 1979), it displays co-operative kinetics with respect to one of its substrates, glucose, though not the other, MgATP (Niemeyer *et al.*, 1975; Storer & Cornish-Bowden, 1976*b*; Tippett & Neet, 1982). This property clearly requires a kinetic explanation, as the classical 'quasi-equilibrium' models of co-operativity (Monod *et al.*, 1965; Koshland *et al.*, 1966) cannot account for the occurrence of co-operativity in a monomeric enzyme with only one binding site for the substrate showing the co-operativity.

All of the current models for kinetic co-operativity assume that it arises from the existence in the mechanism of alternative pathways of reaction that may be slow compared with the reaction as a whole. For example, in the 'mnemonical mechanism' of Ricard *et al.* (1974) the free enzyme can exist in two conformational states that are not at equilibrium and bind substrate with different rate constants; other possibilities are that there may be alternative routes of substrate binding (Ferdinand, 1966; Pettersson, 1986*a*), or there may be recycling of an enzyme–product complex via an enzyme–substrate–product complex (Pettersson, 1986*b*). With any of these mechanisms, but in contrast to classical models of co-operativity, one would expect that the deviations from Michaelis–Menten kinetics could be eliminated by using poorer substrates for which the enzyme–substrate complexes could not be converted into products fast enough to prevent equilibration of substrate binding. In the case of the mnemonical mechanism, which appears to provide a satisfactory explanation of the kinetics of hexokinase D (Storer & Cornish-Bowden, 1977; Pollard-Knight & Cornish-Bowden, 1982), the behaviour expected for a poor analogue of the second substrate is particularly straightforward: as the co-operativity is expected only for the first substrate, but requires rapid binding and reaction of the second, one would expect that replacing the second substrate by a less specific one would decrease the co-operativity. If the lower specificity was a consequence solely of weaker binding it could be overcome by raising the concentration, but if it was a consequence of a slower chemical reaction or slower release of products it could not. We shall show in this paper that MgITP behaves with hexokinase D in the way predicted for an analogue of MgATP that both binds more weakly and reacts more slowly.

MATERIALS AND METHODS

Enzyme

Hexokinase D was purified to a specific activity of 2.5 kat/kg from the livers of well-fed Wistar rats, essentially by the method of Holroyde *et al.* (1976), and was stored at −20 °C in 0.3 M-KCl/1 mM-dithiothreitol/50 mM-glucose/30% (w/v) glycerol/20 mM-triethanolamine/HCl buffer, pH 7.0. Before use it was desalted by passage down a Sephadex G-25 column (10 cm × 0.8 cm) that had been equilibrated with 20 mM-triethanolamine/HCl buffer, pH 7.0, containing 100 mM-KCl, 1 mM-EDTA, 1 mM-dithiothreitol, 1 mM-glucose and 10% (w/v) glycerol. The most active

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fractions from the column were pooled (except when needed for assays at the lowest substrate concentrations, as described below) and kept on ice until required. The concentration of enzyme in the pooled fractions was determined from the measured activity at pH 8.0 and 30 °C in 50 mM-glycylglycine/KOH containing 100 mM-KCl, 1 mM-dithiothreitol, 100 mM-glucose, 0.5 mM-$NADP^+$, 6 mM-$MgCl_2$ and 5 mM-ATP by the glucose 6-phosphate coupled assay with glucose 6-phosphate dehydrogenase as coupling enzyme (Storer & Cornish-Bowden, 1974).

Other materials

ATP (disodium salt), ITP (disodium salt), glycylglycine, dithiothreitol, $NADP^+$, glucose-6-phosphate dehydrogenase (type VIII from baker's yeast) and 99.8 atom% ^2H_2O were obtained from Sigma Chemical Co.

Stock solutions

The concentration of glucose was determined by the ferricyanide assay of Park & Johnson (1949). ITP concentrations were determined by measuring the absorbance at 248.5 nm, assuming a molar absorbance of 12200 (Beaven *et al.*, 1955). The total Mg^{2+} concentration was maintained in constant 1 mM excess over the total ITP concentration: assuming that the stability constants of ITP are similar to those of ATP, this excess is sufficient to ensure that the MgITP concentration is close to 85% of the total ITP concentration over the range of conditions used (Storer & Cornish-Bowden, 1976*a*). Fresh stock solutions of ITP were made each day to avoid problems of instability.

Assays with MgITP as substrate

Assays were carried out at 30 °C in 50 mM-glycylglycine/KOH buffer at pH 8.0, containing 0.1 M-KCl, 1 mM-dithiothreitol, 0.5 mM-$NADP^+$, glucose-6-phosphate dehydrogenase (10 nkat), glucose and MgITP in a total volume of 0.6 ml. When very low substrate concentrations were used very concentrated stock solutions of enzyme were needed to give measurable rates. In such assays the fraction of highest activity from the Sephadex G-25 column was used, enzyme from the other fractions being used for assays at higher substrate concentrations. The differences in enzyme activity were always allowed for in correlating the results.

The glucose added with the enzyme was always corrected for and the enzyme stability was checked periodically during the course of each series of assays. The inclusion of 1 mM-glucose in the desalting buffer was valuable for stabilizing the enzyme and did not prevent assays at very low glucose concentrations, because no more than 0.01 ml of enzyme stock was added to the assay volume of 0.6 ml.

Assays in ^2H_2O

Assays in ^2H_2O were carried out as above, assuming the pL (generalized pH) of solutions in ^2H_2O to be greater by 0.4 than the reading from the pH meter (Glasoe & Long, 1960).

RESULTS

Dependence of the rate on the MgITP concentration

When the MgITP concentration was varied over the range 0.5–15 mM at various glucose concentrations (Fig. 1), the reaction followed Michaelis–Menten kinetics with respect of MgITP. Although MgITP resembles MgATP as substrate in this respect, there are major differences in detail. The apparent K_m for MgITP is highly dependent on the glucose concentration, increasing from 4.5 mM at 1 mM-glucose to 11 mM at 50 mM-glucose, this latter value being about 24-fold higher than the value for MgATP at the same glucose concentration; moreover, except at very low glucose concentrations, below 1 mM, the apparent K_m for MgATP is virtually independent of the glucose concentration (Storer & Cornish-Bowden, 1976*b*). The limiting rate V for MgITP was only 12% of that for MgATP, in accordance with the observation of Parry & Walker (1966) that MgITP is a poor substrate for the enzyme.

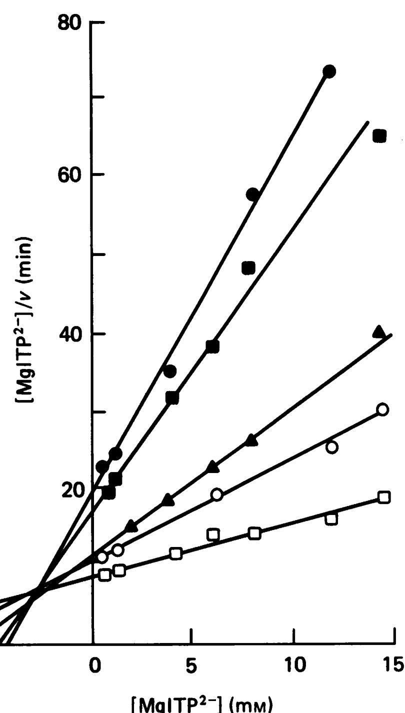

Fig. 1. Hexokinase D kinetics with MgITP as phosphate donor

Initial rates were measured at pH 8.0 for MgITP concentrations in the range 0.5–15 mM as indicated, and 1 mM-(●), 1.5 mM-(■), 5 mM-(▲), 10 mM-(○) or 50 mM-(□) glucose.

Dependence of the rate on the glucose concentration

When the glucose concentration was varied in the range 0.5–25 mM at various concentrations of MgITP (Fig. 2), there was no sign of the positive co-operativity that is the most striking feature of the kinetics when MgATP is the phosphate donor (Storer & Cornish-Bowden, 1976*b*). Even at 20 mM-MgITP the reaction obeys Michaelis–Menten kinetics. This concentration is only about double the apparent K_m for MgITP, so one might argue that it was insufficient to allow MgITP to bind fast enough to prevent equilibration of the enzyme–glucose complex with the two forms of free enzyme postulated by the mnemonical mechanism to exist. However, with MgATP as the phosphate donor, glucose co-operativity is evident not only at MgATP concentrations greater than 2 K_m but remains easily

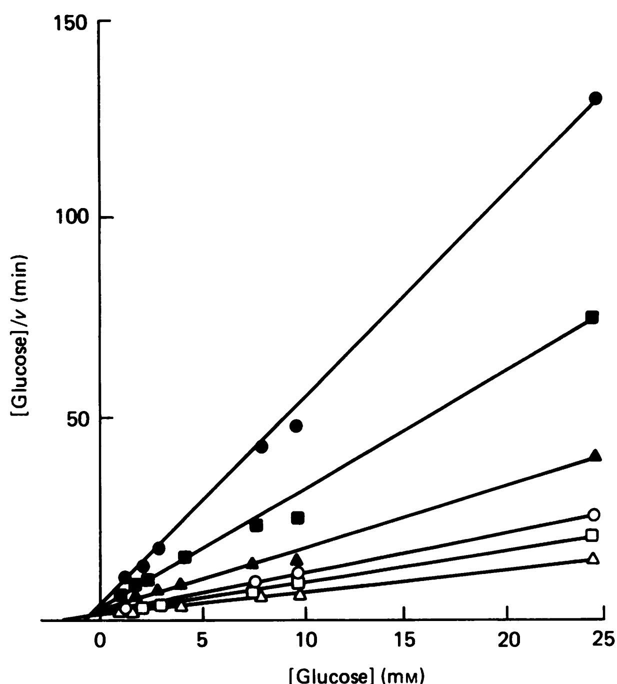

Fig. 2. Lack of glucose co-operativity for hexokinase D with MgITP as phosphate donor

Initial rates were measured at pH 8.0 for glucose concentrations in the range 0.5–25 mM as indicated, and 1.5 mM- (●), 2.5 mM- (■), 5.5 mM- (▲), 8.0 mM- (○), 14.0 mM- (□) or 20.0 mM- (△) MgITP.

detectable at concentrations as low as 0.11 mM, i.e. 0.2 K_m (Storer & Cornish-Bowden, 1976*b*).

Solvent isotope effects

Fig. 3 shows the solvent isotope effects observed with variable glucose concentrations at MgITP concentrations of 4.3 mM (Fig. 3*a*) and 17.2 mM (Fig. 3*b*). The reaction obeys Michaelis–Menten kinetics with respect to glucose in both solvents, again in contrast with the results with MgATP as phosphate donor (Pollard-Knight & Cornish-Bowden, 1984), for which negative co-operativity with respect to glucose was observed in ^2H_2O. This negative co-operativity was also consistent with the mnemonical mechanism, and also required the chemical reaction to be fast enough to prevent equilibration of glucose binding (Cornish-Bowden & Pollard-Knight, 1985), and so its disappearance when the steps following glucose binding are retarded by use of a poor phosphate donor is quite consistent with earlier interpretations.

For MgATP as phosphate donor (Pollard-Knight & Cornish-Bowden, 1984), an inverse isotope effect at low glucose concentrations (i.e. a faster reaction in ^2H_2O than in ^1H_2O) was transformed into a small normal isotope effect at moderate and high glucose concentrations, similar behaviour being observed at all concentrations of MgATP. This is also evident for MgITP as phosphate donor at low MgITP concentrations (Fig. 3*a*), but at high MgITP concentrations (Fig. 3*b*) the lines are virtually parallel (i.e. there is virtually no isotope effect on the limiting rate), and there is an inverse isotope effect over the whole range of glucose concentrations.

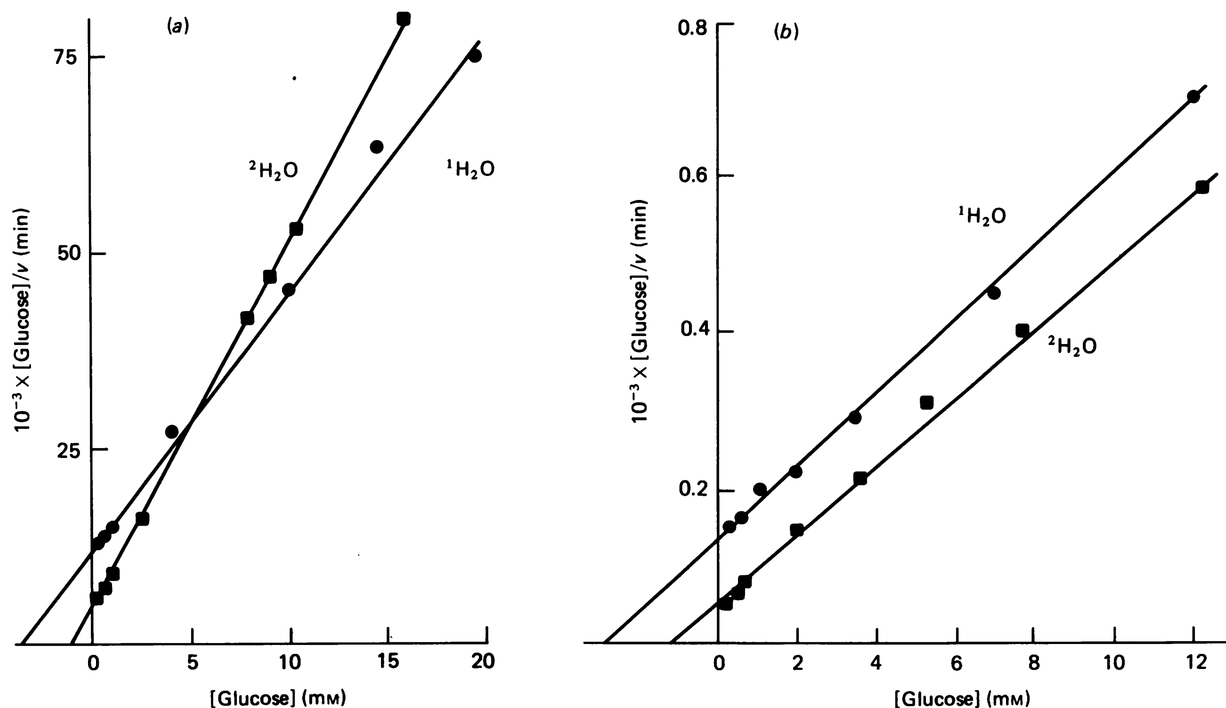

Fig. 3. Solvent isotope effects

Initial rates were measured at pL 8.0 in ^1H_2O (●) or ^2H_2O (■) at glucose concentrations in the range 0.1–20 mM and (*a*) 4.3 mM- or (*b*) 17.2 mM-MgITP.

DISCUSSION

In contrast with the detailed studies of the ATP binding site of yeast hexokinase that have been carried out with ATP analogues with structural alterations in the adenine and ribose rings and the glycosidic linkage of ATP (Hohnadel & Cooper, 1972), very little information of this kind is available for the mammalian hexokinases. Moreover, most of the studies with ATP analogues date from a time when the mammalian isoenzymes were not available in a purified state. Grossbard & Schimke (1966) found that ATP was the only good phosphate donor for hexokinases A, B and C (the isoenzymes with a high affinity for glucose), ITP being a poor analogue of ATP and other nucleoside triphosphates ineffective. Crude preparations of hexokinase D from rabbit liver (Salas *et al.*, 1965) or rat liver (Parry & Walker, 1966) proved to have very low activity with ITP as phosphate donor. Our results with purified hexokinase D are consistent with this, as a 24-fold increase in K_m coupled with an 8-fold decrease in V for MgITP compared with MgATP indicate that at equivalent concentrations MgITP should react about 200 times more slowly than MgATP, so that its capacity to act as substrate is not easily detected.

This high specificity for ATP contrasts with a much lower specificity for glucose, which has recently been studied in detail by Cárdenas *et al.* (1984*a*) and has confirmed a growing conviction that the traditional name of 'glucokinase' is completely inappropriate for hexokinase D. Nonetheless, the availability of MgITP as a poor analogue of MgATP permits a valuable check on the interpretation of the glucose co-operativity as a purely kinetic phenomenon. The fact that the limiting rate is decreased by almost an order of magnitude is important for this purpose, because if the lower specificity for MgITP were simply the consequence of weaker or slower binding its kinetic differences from MgATP could, in principle, be overcome by increasing the MgITP concentration. However, the mnemonical model makes a straightforward prediction that a decrease in the rate of the chemical transformation and release of products should result in loss of co-operativity, whether positive or negative. The fact that the positive co-operativity in ^1H_2O and the negative co-operativity in ^2H_2O are both lost when MgITP is the phosphate donor is thus very satisfactory. There would be no reason to expect loss of co-operativity for one substrate on replacement of the other by a poor analogue if the co-operativity in kinetic experiments merely paralleled the equilibrium behaviour: indeed, Teipel & Koshland (1970) used acetaldehyde as a poor analogue of glyceraldehyde 3-phosphate to monitor the binding of NAD^+ to glyceraldehyde-3-phosphate dehydrogenase; they found that the NAD^+ binding as measured kinetically agreed closely with its behaviour in measurements at true equilibrium.

Although the kinetics with MgITP provide strong support for a kinetic interpretation of the normal co-operativity of hexokinase D, independent of the evidence that the enzyme is monomeric, they do not provide a basis for discriminating between different kinetic models of co-operativity, because all of these require the possibility that the chemical transformation and release of products can occur fast enough to prevent equilibration of substrate binding. This is unfortunate, as discrimination between these models is difficult, and Pettersson (1986*a*,*b*) has recently argued that other models can explain the behaviour of hexokinase D as satisfactorily as the mnemonical mode; he argued similarly for other enzymes for which the mnemonical mechanism had been invoked, such as wheat-germ hexokinase L_I (Meunier *et al.*, 1974) and octopine dehydrogenase (Monneuse-Doublet *et al.*, 1978). Nonetheless, as discussed elsewhere (Cornish-Bowden & Storer, 1986), these other models are not without problems, as they predict behaviour that is not observed, such as deviations from Michaelis–Menten kinetics with respect to MgATP. The results reported here may be added to a growing body of experimental data that are consistent with a model involving slow interconversion of enzyme forms. This may be a mnemonical mechanism of the type originally described by Ricard *et al.* (1974), in which the conformational transition occurs only at the level of free enzyme; or it may be a slow-transition model of the form developed by Ainslie *et al.* (1972), in which enzyme–substrate complexes also exist in multiple states. This more general development from the same fundamental ideas has been preferred by some workers (Tippett & Neet, 1982; Cárdenas *et al.*, 1984*b*) as an interpretation of the kinetic behaviour of hexokinase D.

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REFERENCES

Ainslie, G. R., Shill, J. P. & Neet, K. E. (1972) J. Biol. Chem. **247**, 7088–7096

Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955) in The Nucleic Acids (Chargaff, E. & Davidson, J. N., eds.), vol. 1, pp. 493–553, Academic Press, New York

Cárdenas, M. L., Rabajille, E. & Niemeyer, H. (1984*a*) Biochem. J. **222**, 363–373

Cárdenas, M. L., Rabajille, E. & Niemeyer, H. (1984*b*) Eur. J. Biochem. **145**, 163–171

Connolly, B. A. & Trayer, I. P. (1979) Eur. J. Biochem. **99**, 299–308

Cornish-Bowden, A. & Pollard-Knight, D. (1985) Arch. Biol. Med. Exp. **18**, 293–300

Cornish-Bowden, A. & Storer, A. C. (1986) Biochem. J. **240**, 293–296

Ferdinand, W. (1966) Biochem. J. **98**, 278–283

Glasoe, P. K. & Long, F. A. (1960) J. Phys. Chem. **64**, 188–190

Grossbard, L. & Schimke, R. T. (1966) J. Biol. Chem. **241**, 3456–3560

Hohnadel, D. G. & Cooper, C. (1972) Eur. J. Biochem. **31**, 180–185

Holroyde, M. J., Allen, M. B., Storer, A. C., Warsy, A. S., Chesher, J. M. E., Trayer, I. P., Cornish-Bowden, A. & Walker, D. G. (1976) Biochem. J. **153**, 363–373

Koshland, D. E., Jr., Némethy, G. & Filmer, D. (1966) Biochemistry **5**, 365–385

Meunier, J.-C., Buc, J., Navarro, A. & Ricard, J. (1974) Eur. J. Biochem. **49**, 209–223

Monneuse-Doublet, M. E., Olomucki, A. & Buc, J. (1978) Eur. J. Biochem. **84**, 441–448

Monod, J., Wyman, J. & Changeux, J.-P. (1965) J. Mol. Biol. **12**, 88–118

Niemeyer, H., Cárdenas, M. L., Rabajille, E., Ureta, T., Clark-Turri, L. & Peñaranda, J. (1975) Enzyme **20**, 321–333

Park, J. T. & Johnson, M. J. (1949) J. Biol. Chem. **181**, 149–151

Parry, M. J. & Walker, D. G. (1966) Biochem. J. **99**, 266–274

Pettersson, G. (1986*a*) Biochem. J. **233**, 347–350

Pettersson, G. (1986*b*) Eur. J. Biochem. **154**, 167–170

Pollard-Knight, D. & Cornish-Bowden, A. (1982) Mol. Cell. Biochem. **44**, 71–80

Pollard-Knight, D. & Cornish-Bowden, A. (1984) Eur. J. Biochem. **141**, 157–163

Ricard, J., Meunier, J.-C. & Buc, J. (1974) Eur. J. Biochem. **49**, 195–208

Salas, J., Salas, M., Viñuela, E. & Sols, A. (1965) J. Biol. Chem. **240**, 1014–1018

Storer, A. C. & Cornish-Bowden, A. (1974) Biochem. J. **141**, 205–209

Storer, A. C. & Cornish-Bowden, A. (1976*a*) Biochem. J. **159**, 1–5

Storer, A. C. & Cornish-Bowden, A. (1976*b*) Biochem. J. **159**, 7–14

Storer, A. C. & Cornish-Bowden, A. (1977) Biochem. J. **165**, 61–69

Teipel, J. & Koshland, D. E., Jr. (1970) Biochim. Biophys. Acta **198**, 183–191

Tippett, P. S. & Neet, K. E. (1982) J. Biol. Chem. **257**, 12846–12852

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