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# Mechanistic origin of the sigmoidal rate behaviour of rat liver hexokinase D ('glucokinase')

Athel CORNISH-BOWDEN* and Andrew C. STORER†

*Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile, and †Division of Biological Sciences, National Research Council, Ottawa, Ont. K1A 0R6, Canada

Two recent proposals to account for the kinetic co-operativity of hexokinase D ('glucokinase') from rat liver are examined. A model in which the deviations from Michaelis–Menten kinetics result from a random order of binding of the substrates [Pettersson (1986) Biochem. J. **233**, 347–350] accounts satisfactorily for the behaviour as a function of glucose concentrations, but it also predicts observable substrate inhibition by MgATP, which is in fact not observed. An alternative proposal in which the deviations arise from recycling of an enzyme–MgADP complex [Pettersson (1986) Eur. J. Biochem. **154**, 167–170] also accounts satisfactorily for some of the data, but the required enzyme–MgADP complex could not be detected in isotope-exchange measurements. Thus the mnemonical mechanism proposed originally [Storer & Cornish-Bowden (1977) Biochem. J. **165**, 61–69], which explains the deviations in terms of a relatively slow interconversion between two forms of free enzyme, remains the most parsimonious explanation of the behaviour of hexokinase D.

## INTRODUCTION

Hexokinase D (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1; formerly commonly known as 'glucokinase', EC 2.7.1.2) is the hexokinase isoenzyme characteristic of the liver of vertebrates. Despite existing as a monomeric protein of about 48 kDa (Holroyde *et al.*, 1976), it displays sigmoidal kinetics with respect to the concentration of one substrate, glucose, but Michaelis–Menten kinetics with respect to the other, MgATP (Niemeyer *et al.*, 1975; Storer & Cornish-Bowden, 1976; Tippett & Neet, 1982). We (Storer & Cornish-Bowden, 1977) interpreted these and other properties of hexokinase D in terms of the 'mnemonical' mechanism developed originally by Ricard *et al.* (1974) to account for the negative co-operativity of hexokinase $L_I$ from wheat germ (Meunier *et al.*, 1974). The essence of this mechanism is that the free enzyme can exist in two states that differ in affinity for glucose and are interconverted sufficiently slowly that the isomerization is not necessarily close to equilibrium under steady-state conditions; an important proviso is that the substrate that shows deviations from Michaelis–Menten kinetics should bind to the enzyme before the substrate that does not.

Subsequent investigations (Gregoriou *et al.*, 1981; Pollard-Knight & Cornish-Bowden, 1984) have in general been consistent with this interpretation of the kinetics, and workers in other laboratories have reached similar conclusions (Tippett & Neet, 1982; Cárdenas *et al.*, 1984). In two recent papers, however, Pettersson (1986*a*,*b*) has argued that the deviations from Michaelis–Menten kinetics can equally well be explained in terms of a random-order ternary-complex mechanism (Pettersson, 1986*a*) or a compulsory-order mechanism in which one enzyme–product binary complex (enzyme–MgADP) can be recycled via a non-productive enzyme–substrate–product complex (enzyme–glucose–MgADP) (Pettersson, 1986*b*). In neither case does he consider enzyme isomerization to be necessary, and he also suggests that the latter mechanism may account for the kinetics of wheat-germ hexokinase $L_I$. These proposals by Pettersson (1986*a*,*b*) are interesting and include various points that will need to be given more attention in the future than they have received hitherto. Nonetheless, as explanations of the kinetics of hexokinase D they create difficulties that the mnemonical mechanism avoids, and for that reason we continue to regard the mnemonical mechanism as the most economical hypothesis to account for the data.

## THEORY AND RESULTS

### Random-order ternary-complex mechanism

In our original discussion of the interpretation of the kinetics of hexokinase D (Storer & Cornish-Bowden, 1977), we recognized that a random-order mechanism could account for the deviations from Michaelis–Menten kinetics, and that if the terms in $[MgATP]^2$ required by it were negligible in the experimental range of concentrations the predicted kinetic behaviour would be indistinguishable on the basis of initial-rate measurements from that predicted by the mnemonical mechanism. Nonetheless, we considered that 'the mnemonical model is more attractive, for several reasons. In view of the absence of any perceptible curvature in the primary plots for $MgATP^{2-}$ (Storer & Cornish-Bowden, 1976) it would be artificial to argue the presence of undetected terms in $[MgATP^{2-}]^2$ in the rate equation. Moreover, the mnemonical model is simpler, and it accounts very neatly for the decrease in glucose co-operativity at low $MgATP^{2-}$ concentrations.'

The subsequent demonstration that glucose binds before MgATP under steady-state conditions (Gregoriou

* On leave of absence from Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K., to which address correspondence should be directed.

*et al.*, 1981), and that no enzyme–MgATP binary complex could be detected, seemed to confirm the validity of the mnemonical mechanism and to rule out the random-order mechanism. Although isotope-exchange measurements at equilibrium (Gregoriou *et al.*, 1981) indicated some participation of an alternative pathway with MgATP binding before glucose, its contribution appeared to be too slight to account for the deviations from Michaelis–Menten kinetics. Pettersson (1986*a*) makes a valuable point, therefore, in noting that 'theoretical analyses have failed to reveal any intuitively obvious relationship between the rate behaviour of and partitioning of reaction flow in random ternary-complex mechanisms (Pettersson, 1969; Andersson *et al.*, 1984)', and that 'observations indicating that one of the alternative pathways for ternary-complex formation ... is of little significance with respect to net reaction flow cannot be taken to exclude that this pathway may contribute most significantly to the higher-degree behaviour of the system.'

Nonetheless, we cannot agree with Pettersson (1986*a*) that the plots of [glucose]/rate against [glucose] shown in Fig. 1 of his paper 'establish that the [random-order] model does provide a most satisfactory fit to the ... data.'. It is true that his model, with the parameter values that he tabulates, provides a superficially acceptable description of the glucose kinetics. This, however, was never at issue (cf. Storer & Cornish-Bowden, 1977). The difficulty with the random-order model is not that it fails to account for the glucose kinetics but that it fails to provide a satisfactory explanation of the MgATP kinetics without resorting to assumptions about the presence of undetected terms in the rate equation. In any case, with the parameter values given by Pettersson (1986*a*), these higher-order terms would not be undetected, because according to his equation substrate inhibition by MgATP should have been sufficiently pronounced to be readily detectable, at least at the lower glucose concentrations.

According to the equation and parameter values given by Pettersson (1986*a*), the rate should have been a maximum at 3.8 mM-MgATP for the experiment with 1 mM-glucose, and at MgATP concentrations of 5.5 mM, 8.1 mM, 13.1 mM and 51.7 mM in the experiments at 2.5 mM-, 5 mM-, 10 mM- and 50 mM-glucose respectively. As the highest MgATP concentration actually used was 8.6 mM at each of the five glucose concentrations, it follows that the maxima ought to have been within the experimental ranges for the data at the three lowest glucose concentrations. Plotting the original data at 1 mM-glucose in the region where substrate inhibition should have been clearly visible, and using an expanded scale to emphasize any random error, we obtain Fig. 1, from which a systematic failure to follow the behaviour predicted by Pettersson's (1986*a*) model is evident whereas the mnemonical model (broken line) fits satisfactorily. This plot includes two observations, at 6.5 mM- and 8.6 mM-MgATP, that were omitted from Fig. 4 of Storer & Cornish-Bowden (1976) in order to avoid undue compression of the scale, but even if these points are ignored the systematic deviations from the line calculated for Petterson's (1986*a*) model remain evident. In corresponding plots of the data at 2.5 mM- and 5 mM-glucose (not shown) there was similar though less pronounced behaviour. At 10 mM- and 25 mM-glucose systematic error could not be distinguished from random

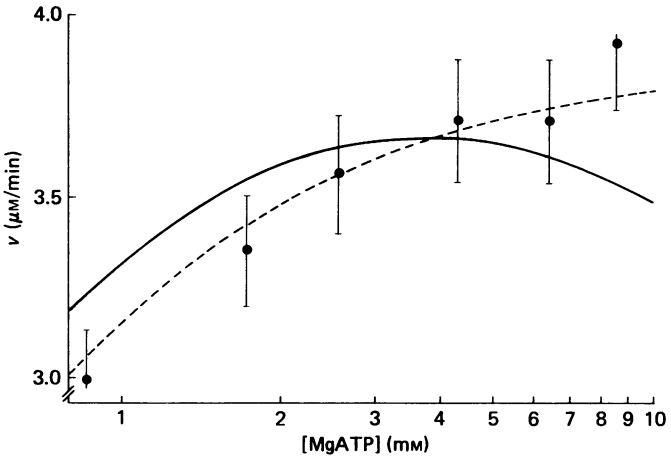


**Fig. 1. Lack of substrate inhibition by MgATP under conditions where it is predicted by the random-order model**

The Figure shows data from Fig. 4 of Storer & Cornish-Bowden (1976), with the inclusion of two additional observations at the highest MgATP concentrations that were off the scale of the original, for the reaction catalysed by hexokinase D from rat liver at 30 °C and pH 8.0 in the presence of 1 mM-glucose. To facilitate distinction between random experimental error and lack of fit to the model proposed by Pettersson (1986*a*), an expanded scale is used for the ordinate and only data in the vicinity of the putative maximum are shown. The continuous line is calculated from the random-order model by using the parameter values tabulated by Pettersson (1986*a*), scaled so as to give a mean of zero for the percentage deviation of the plotted points from the line. Note, however, that the individual values of the percentage deviation increase monotonically with the MgATP concentration. The broken line is calculated from eqn. (2) of Storer & Cornish-Bowden (1977) by using the parameter values given in Table 2 of the same paper, scaled in the same way as for the random-order model. The error bars are calculated for $\pm 4.6\%$, this value being the square root of the mean square for pure error given in Table 3 of Storer & Cornish-Bowden (1977).

error, but this is hardly surprising as in these cases the predicted maxima were well outside the experimental range.

We may add that other experimenters have also found that hexokinase D obeys Michaelis–Menten kinetics with respect to MgATP (Niemeyer *et al.*, 1975; Tippett & Neet, 1982). Even in experiments at MgATP concentrations as high as 20 mM (Cárdenas *et al.*, 1979), no deviations from Michaelis–Menten kinetics were noted.

Pettersson (1986*a*) makes the further remarkable observation that the higher-order effects of a pathway via an enzyme–MgATP complex do not disappear even if the rate constants involving this complex are decreased indefinitely: 'the pronounced deviations from Michaelis–Menten kinetics will persist however minute a contribution the $ES_2$ pathway is assumed to provide'. Although this is true, it should be interpreted with caution, because as the rate constants are decreased the time required to reach the steady state must increase concomitantly, eventually becoming large compared with the time scale of the experiment. This time will be of the order of $3/(k_{+2}[\text{MgATP}]+k_{+4}[\text{glucose}]+k_{-2}+k_{-4})$, about 0.2 s at 5 mM-MgATP and 2.5 mM-glucose for the values of

the rate constants suggested by Pettersson (1986*a*). This is small compared with the time scale of our original experiments, but the rate constants involving the enzyme–MgATP binary complex could not be decreased by more than a factor of about 300-fold without introducing readily observable non-linearity in the progress curves.

### Cycling through a non-productive complex

The second proposal made by Pettersson (1986*b*) to account for the kinetics of hexokinase B is quite different. Here, he suggests that the binding of substrates may indeed follow a compulsory order, with glucose binding before MgATP, but with a random-order release of products. If one of the binary enzyme–product complexes, enzyme–MgATP, is capable of binding glucose and then releasing MgATP, it can allow enzyme to be recycled that might otherwise be sequestered as a slowly dissociating enzyme–product complex. Such cycling would generate a higher-order dependence on the glucose concentration, and, unlike the random-order mechanism, would imply no higher-order dependence on the MgATP concentration. Superficially, therefore, it would appear to be a more attractive hypothesis to explain the behaviour of hexokinase D than the random-order mechanism.

Although one might object that it is inherently implausible to propose that substrate binding follows a compulsory order whereas products can be released in either order, this is not an essential feature of the model. Earlier study of a similar model in which substrates bound in either order in a rapid equilibrium (Storer, 1975) showed similar properties to those in the model as given by Pettersson (1986*b*). In the following discussion, however, we shall consider the model in the latter form.

Recycling of an enzyme–product complex via a non-productive complex is an interesting idea that does not appear to have been considered previously in published work as a mechanism for generating deviations from Michaelis–Menten kinetics. Moreover, it accounts for our original data for hexokinase D almost as well as the mnemonical model (Storer, 1975). Nonetheless, in relation to more recent data it presents a serious difficulty, because it implies a major role for an enzyme–MgADP complex, such that under some conditions (low glucose with high MgATP concentrations) it may be the most abundant enzyme form apart from the free enzyme. Yet both trapping experiments and flux-ration experiments failed to show any evidence for such a complex (Gregoriou *et al.*, 1981), though there was no difficulty about detecting the existence of the other possible enzyme–product binary complex, enzyme–glucose 6-phosphate.

## DISCUSSION

There is considerable overlap between the types of kinetic behaviour predicted by the various models that can account for kinetic co-operativity, and definitive distinction between them is always likely to be difficult and to require examination of various different kinds of experiment. In the case of hexokinase D, these have included, in our laboratory, initial-rate measurements (Storer & Cornish-Bowden, 1976), product inhibition (Storer & Cornish-Bowden, 1977), isotope exchange both at equilibrium and in the steady state (Gregoriou *et al.*, 1981), and solvent isotope effects (Pollard-Knight & Cornish-Bowden, 1984). More chemical approaches, such as affinity labelling (Connolly & Trayer, 1979) and direct structural measurements, which would be very useful in establishing whether the two states of free enzyme required by the mnemonical model exist, have been limited by the lack of a procedure for obtaining the enzyme in reagent quantities.

One is forced, therefore, to rely on plausibility arguments to decide whether, as maintained by Pettersson (1986*b*), 'the rate behaviour of all enzymes hitherto proposed to operate by a mnemonical mechanism can presently be more readily explained in terms of substrate binding to differently ligated states of the enzyme.'. For example, it is striking that the rate constants proposed by Pettersson (1986*a*) for the random-order mechanism require the free enzyme to bind glucose about 70 times more weakly than it binds MgATP, but to do so about 1200 times more rapidly; on the other hand, glucose binds to the enzyme–MgATP complex about 1300 times more slowly than MgATP binds to the enzyme–glucose complex, the ratio of equilibrium constants being again about 70. These ratios of rates are lower limits, because Pettersson (1986*a*) considers that the kinetic behaviour would be essentially unchanged if the rate constants involving the enzyme–MgATP complex were made even smaller. Such discrepancies between rate behaviour and equilibrium behaviour are not, of course, impossible, because there is no requirement for thermodynamically favourable processes to occur rapidly. Nonetheless, it is hard to visualize how they could arise without postulating isomerization steps that would be no less objectionable than the existence of two states of free enzyme required by the mnemonical model. Pettersson (1986*b*) does not suggest values for the 'on' rate constants for binding of glucose 6-phosphate and MgADP in the recycling mechanism, but it is likely that similar difficulties would arise if one tried to find values that would explain why it was not possible to detect an enzyme–MgADP complex but easy to detect an enzyme–glucose 6-phosphate complex (Gregoriou *et al.*, 1981). The mnemonical mechanism continues, therefore, to be the most parsimonious hypothesis to account for the kinetic behaviour of hexokinase D.

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