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Mammalian Hexokinases: A System for the Study of Co-operativity in Monomeric Enzymes*

Hexoquinasas de mamíferos: Un sistema para el estudio de la cooperatividad de enzimas monoméricas

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Kinetic and structural studies have been carried out of two isoenzymes of hexokinase from the rat, hexokinase II and glucokinase. Although both enzymes are monomeric, hexokinase II has a molecular weight double that of glucokinase and resembles a dimer of glucokinase. The co-operativity of glucokinase, which is not observed for hexokinase II, appears to be kinetic in origin rather than the consequence of interactions between distinct glucose-binding sites.

HEXOKINASE II / GLUCOKINASE / CO-OPERATIVITY / MONOMERIC ENZYMES

Although the existence of four isoenzymes of hexokinase (EC 2.7.1.1) in mammals has been known for a long time, and in spite of their obvious metabolic importance, study of the mammalian hexokinases has lagged far behind that of the yeast enzymes. The principal reason has been the lack of convenient methods of purification: until recently, only hexokinase I, the main isoenzyme in brain, was conveniently available in a pure state (1). The use of affinity chromatography on N-acetylglucosamine linked to Sepharose has radically improved this state of affairs, and we now have convenient methods for purifying hexokinase II (2), the main isoenzyme in muscle, hexokinase III (unpublished), a minor isoenzyme found in several tissues, and hexokinase IV (3), which is conveniently referred to as glucokinase (EC 2.7.1.2).

Comparison of the structural and catalytic properties of the mammalian hexokinases, especially hexokinase II and glucokinase, shows both structural similarities and important kinetic differences. We believe that understanding of these properties will not only be valuable in the limited context of glucose phosphorylation, but will also lead to a deeper understanding of the nature of co-operativity especially in monomeric systems.

Kinetics of Glucokinase

Studies of glucokinase kinetics by Parry and Walker (4) and González *et al.* (5) revealed deviations from the Michaelis-Menten equation when the rate was measured as a function of glucose concentration. Although the validity of these observations remained in doubt for

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some years, because the deviations were relatively small, subsequent work over a wider range of substrate concentrations confirmed and extended them (6-9). Is is now clear that although the Michaelis-Menten equation is obeyed to within experimental error at glucose concentrations above 10 mM, there are deviations at lower concentrations that become very pronounced when the range is extended to 0.1 mM-glucose. It is convenient to quantify the deviations in terms of the Hill coefficient, h (10), and it is found that the value of h for glucose depends on the concentration of the other substrate, $MgATP^{2-}$. At high $MgATP^{2-}$ concentrations, h has a value of about 1.5, wich can be extrapolated to about 1.6 at saturation; this indicates a moderate degree of positive co-operativity. The value of h decreases as the $MgATP^{2-}$ concentration is decreased: at 0.1 mM-$MgATP^{2-}$ the lowest concentration at which accurate rate measurements are possible, $h = 1.2$, but it seems reasonable to extrapolate the curve to $h = 1.0$ at vanishingly small concentrations of $MgATP^{2-}$; in other words, we believe that the co-operativity disappears in the absence of $MgATP^{2-}$. This disappearance of cooperativity probably has no physiological importance, because h is close to its maximum value and insensitive to $MgATP^{2-}$ at the likely physiological concentration of 2 mM.

In spite of this dependence of the co-operativity for glucose on the concentration of $MgATP^{2-}$, we have detected no co-operativity for $MgATP^{2-}$ itself: plots of $[MgATP^{2-}]/v$ against $[MgATP^{2-}]$ at any glucose concentration in the range 1-50 mM show no perceptible deviations from linearity over the whole range of $MgATP^{2-}$ concentrations studied (0.1 - 10 mM).

Product-inhibition studies of glucokinase have provided rather complex and puzzling results. Most inhibitions are mixed in type, *i.e.* both competitive and uncompetitive components are present; but, with 50 mM-glucose, inhibition by glucose 6-P with $MgATP^{2-}$ as varied substrate proved to be competitive, $K_i = 11$ mM, and no uncompetitive component could be detected. In plots of $[MgATP^{2-}]/v$ against [glucose 6-P], which ought to give an intersecting set of lines for any type of inhibition other than pure competitive (11), the lines were parallel to within experimental error.

Kinetics of Hexokinase II

In contrast to glucokinase, the kinetics of hexokinase II are rather simple (2). The Michaelis-Menten equation is obeyed whichever substrate concentration is varied, with $K_m = 0.2$ mM for glucose and $K_m = 0.8$ mM for $MgATP^{2-}$. Both values are independent of the concentration of the other substrate. No co-operativity is evident either with glucose or with $MgATP^{2-}$.

Structural Studies of Glucokinase and Hexokinase II

The kinetic observations given above, together with the results of structural studies, are given in Table 1. The molecular weight of hexokinase II is double that of glucokinase. This suggests that hexokinase II approximates to a dimer of glucokinase, though in fact both enzymes are monomeric because neither molecular weight is affected by dissociating conditions. The resemblance of hexokinase II to a dimer of glucokinase is strongly supported by the striking similarity between their amino acid compositions. If one halves the numbers of residues of each type in hexokinase II and compares them with the corresponding numbers for glucokinase, one may calculate the index $S\Delta n$ (12), defined as follows:

$$S\Delta n = \frac{1}{2}\sum_{i=1}^{18}(n_{iA} - n_{iB})^2$$

where n_{iA} is the number of residues of the ith type in the first protein (in the present case half of the number for hexokinase II) and n_{iB} is the corresponding number for the second protein (glucokinase), and the summation is over all of the types of residue distinguished in composition studies (ideally 18, but 16 in our case because values for tryptophan and cysteine were not available). The resulting value $S\Delta n = 69$ is an estimate of the number of differences between the sequences. Even allowing for the large statistical uncertainty of this estimate [the coefficient of variation is about 40%, both predicted (12) and observed in studies of proteins of known sequence (13)], it indicates that the sequences are 75%-95% identical.

TABLE 1

Comparison between Hexokinase II and Glucokinase

Property	*Hexokinase II (Rat muscle)*	*Glucokinase (Rat liver)*
Quaternary structure	monomeric	monomeric
Molecular weight	96.000	48.000
Amino acid composition	very similar, suggesting that the sequences are of the order of 85% identical	
Number of glucose sites	1 per monomer	not known
Half-saturation point for glucose	0.2 mM (K_m)	5 mM
Hill coefficient for glucose,		
(a) low $MgATP^{2-}$	1.0	1.2 (1.0?)
(b) high $MgATP^{2-}$	1.0	1.6
K_m for $MgATP^{2-}$	0.8 mM	0.55 mM
Hill coefficient for $MgATP^{2-}$	1.0	1.0
Sensitivity to glucose 6-phosphate	K_i = 0.08 μM	K_i = 60 mM

Affinity Labelling of Hexokinase II

We have studied the inactivation of hexokinase II by N-bromo-acetyl-2-amino-2-deoxy-D-glucopyranose. The inhibition is competitively inhibited by glucose, which leads us to believe that it is a true effect at the active site of the enzyme. Studies with ^{3}H-labelled inhibitor indicate that the inactivation is complete when about 0.7 mol inhibitor is incorporated per mol enzyme. We believe that the correct stoichiometry is 1:1 and that the lower value is caused by the inherent instability of the purified enzyme. We are currently engaged in further experiments to provide similar information for glucokinase, and more accurate data for hexokinase II. The above compound inactivates glucokinase very slowly and we have therefore prepared a more efficient affinity label, N-(N-bromoacetyl-6-amino-hexanoyl)-glucosamine, in which the bromoacetyl group is separated from the pyranose ring by a spacer arm.

Interpretation of Glucokinase Co-operativity

The apparently close structural similarity between glucokinase and hexokinase II makes it very unlikely that the mechanisms of the reactions catalysed by the two enzymes are fundamentally different. The existence of only one specific glucose site per molecule of hexokinase II thus makes it unreasonable the postulate of mechanism for glucokinase that would require interaction between two or more specific glucose sites on a molecule only half as large as the hexokinase II molecule.

We therefore believe that the co-operativity of glucokinase should be explained in terms of a purely kinetic model of the sort proposed originally by Rabin (14) and developed by Ricard *et al.* (15). In this model, shown in Fig 1, only one active site is required, but the enzyme is capable of existing in two states, which bind glucose with different kinetic constants to give the same enzyme-glucose complex. $MgATP^{2-}$ binds only to this complex, and its concentration determines whether equilibrium between the two forms of the free enzyme and the enzyme-glucose complex is possible. At low $MgATP^{2-}$ concentrations the rate of the complete reaction is slow and glucose binding is able to achieve equilibrium; as there is only one site, there can be no co-operativity. At high $MgATP^{2-}$ concentrations the enzyme-glucose complex is removed faster than it can equilibrate with free enzyme and glucose: as a result, terms in $[glucose]^2$ appear in the rate equation and generate co-operativity. In this mechanism, $MgATP^{2-}$ participates in one step only of the reaction; there are therefore no terms in the square of its concentration and no $MgATP^{2-}$ co-operativity.

The product-inhibition data are more difficult to explain, especially the apparent competition between $MgATP^{2-}$ glucose 6-P. To postulate that these species compete for the free enzyme would pose more problems

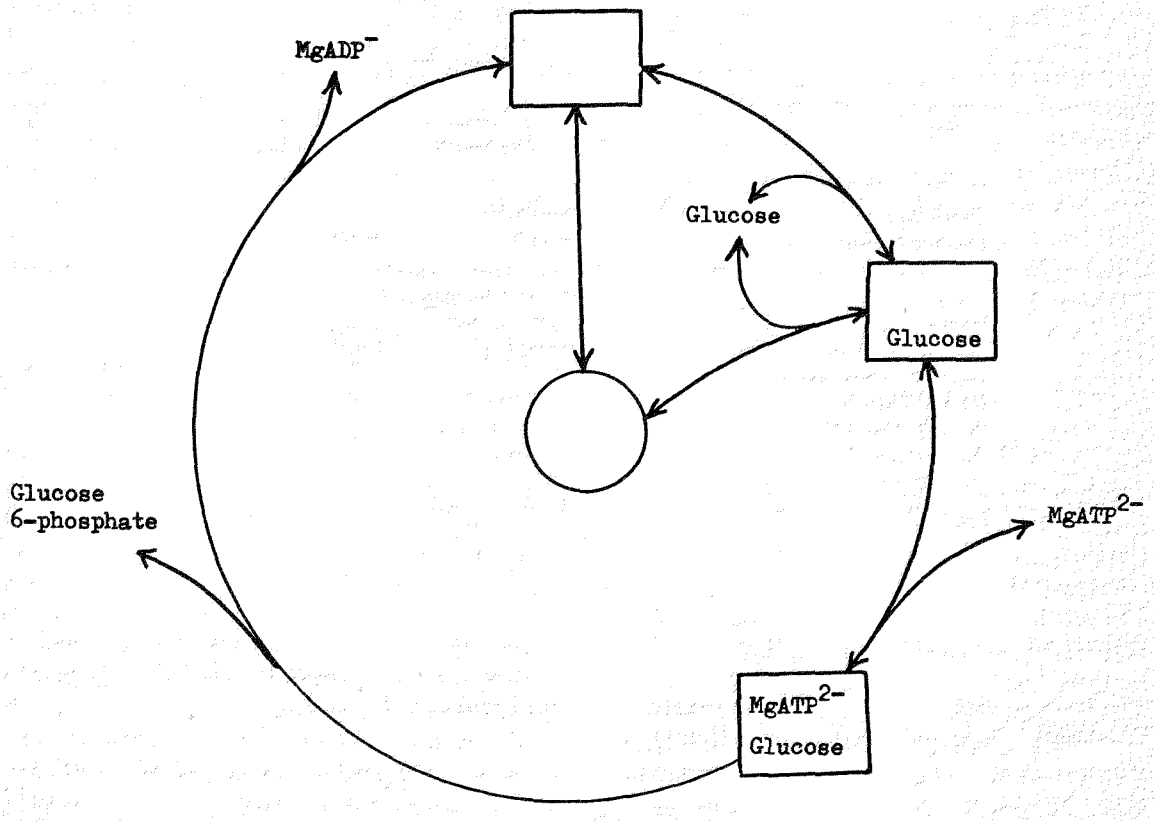

Fig. 1. Mnemonical Model for Glucokinase. The circle and rectangle represent two states of the enzyme with different affinities for glucose.

than it would solve, and we therefore tentatively suggest that glucose 6-P is the first product to be released and that the inhibition is competitive because the displacement of glucose 6-P by $MgATP^{2-}$ is virtually a concerted reaction, with no appreciable build-up of ternary complex. Such a concerted reaction, as in the 'Theorell-Chance mechanism' (16), does in principle lead to competitive inhibition (see Cornish-Bowden, 10), but we are not aware of other experimental examples.

The structural similarity between glucokinase and hexokinase II suggest that there ought to be two active sites on each molecule of the larger enzyme, rather than the one indicated by the affinity-labelling experiments. We suggest that hexokinase II occurred originally as a true dimer gene duplication, but that it has subsequently evolved into a monomeric structure in which one of the original active sites has become an allosteric binding site to provide the physiologically valuable inhibition by glucose 6-P. This would explain why glucokinase shows no hint of comparable allosteric inhibition by glucose 6-P, with a K_i value nearly a million times larger than the value for hexokinase II.

The model for glucokinase co-operativity predicts that a less efficient substrate than $MgATP^{2-}$ would also be less efficient than $MgATP^{2-}$ in promoting glucose co-operativity. We are currently testing this prediction with $MgITP^{2-}$, which is about 30 times poorer than $MgATP^{2-}$ as a substrate for glucokinase. Preliminary results are in accordance with those predicted, *i.e.* we observe no glucose co-operativity when $MgITP^{2-}$ is the second substrate, but the low rates observed with this system have so far made it difficult to make accurate enough measurements for firm conclusions to be drawn.

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