STUDIES ON THE KINETICS AND MECHANISM OF ORTHOPHOSPHATE ACTIVATION OF BOVINE BRAIN HEXOKINASE*

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SUMMARY: The binding of glucose to bovine brain hexokinase, isozyme I, exhibited one binding site per 100,000 molecular weight. Glucose-6-P binding was examined in the absence and presence of ATP. ATP and glucose-6-P were shown to compete for the same binding site on the enzyme. A model was proposed to account for these findings and the previously reported data that glucose-6-P and P_i exhibit mutually exclusive, non-cooperative binding to the enzyme. The model shows that brain hexokinase exists in two rapidly interconvertible states, either with or without P_i and that glucose-6-P binding to the phosphate associated enzyme form is relatively very poor. This proposal has been tested kinetically and the data appear to support the suggested model.

In a recent report we suggested a mechanism for orthophosphate (P_i) activation of bovine brain hexokinase (Type I isozyme) based upon studies of ligand binding (1). Using the Paulus binding technique (2), and hexokinase prepared according to the method of Redkar and Kenkare (3), we found that the enzyme binds one mole of glucose-6-P or P_i per mole of enzyme (M.W. 98,000). These experiments also demonstrated clearly that glucose-6-P and P_i could not be bound to hexokinase simultaneously; i.e., binding was mutually exclusive. No evidence for cooperative binding was found from these studies.

It has been recognized for some time that glucose-6-P is a normal competitive product inhibitor of ATP in the brain hexokinase reaction (4-7). It is also known that P_i does not activate brain hexokinase in the absence of glucose-6-P inhibition (8), nor does P_i affect the Km for ATP and

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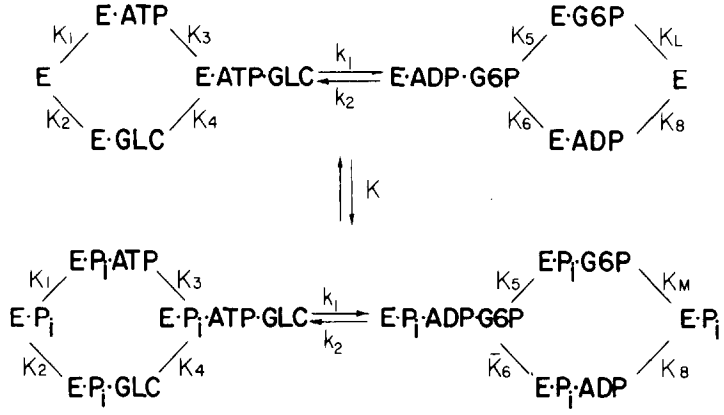
glucose or the Vm of the reaction (7,8). These observations along with the idea that the kinetic mechanism of the brain hexokinase reaction is of the rapid equilibrium Random Bi Bi type (8-10), led to the model shown in Scheme I. The rate expression for the model is presented in Equation 1.



SCHEME I

$$\frac{E_o}{v} = \phi_o + \frac{\phi_{ATP}}{(ATP)} + \frac{\phi_{GLC}}{(GLC)} + \frac{\phi_{ATP\cdot GLC}}{(ATP)(GLC)}\left[1 + \frac{(G6P)}{K_L}\cdot\frac{K_M K + K_L(P_i)}{K_M(K+(P_i))}\right] \qquad (1)$$

The purpose of this report is to present additional data on ligand binding to brain hexokinase. In addition, kinetic experiments were carried out in an attempt to correlate P_i reversal of glucose-6-P inhibition and the proposed activation mechanism. Data are also presented which suggest that the method of brain hexokinase preparation is a factor which must be considered in the evaluation of the kinetics of hexokinase enzymology.

MATERIALS AND METHODS: D-Glucose was purchased from Calbiochem. ATP, and glucose-6-P were products of Sigma. [U-^{14}C]-D-Glucose (specific acitivity, 162 mCi per mmole) and enzyme grade Tris base were supplied by Schwarz/Mann. Diaflo membranes, PM 10, were purchased from Amicon.

Bovine brain hexokinase prepared by the method of Redkar and Kenkare (3) was judged homogeneous with disc-gel electrophoresis. The specific activity

of this preparation was 66 units per mg under the standard assay conditions (11).

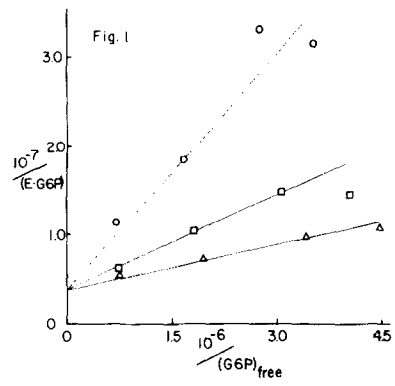
[U-^{14}C] - D-Glucose-6-P was prepared from [U-^{14}C] -D-glucose utilizing yeast hexokinase and purified on Dowex 1 (HCO_3^-).

All other experimental details are described in the Figure Legends.

RESULTS AND DISCUSSION: We recently found that brain hexokinase binds one molecule of glucose-6-P or one P_i molecule per molecule of enzyme of molecular weight 98,000 (1). The kinetic mechanism is rapid equilibrium Random Bi Bi (8-10) and P_i is thought not to either alter the Vm or the Km's for ATP and glucose. We have observed that P_i is a competitive inhibitor of glucose-6-P in binding experiments (1) and this observation correlated well with the known activation effects of P_i on brain hexokinase in the presence of glucose-6-P (7,8). Wilson (12), in a footnote to a recent paper finds very similar stoichiometry of ligand binding.

Although kinetic studies have indicated that glucose-6-P is a normal competitive product inhibitor of ATP with brain hexokinase (4-7), it has been suggested that glucose-6-P is an allosteric inhibitor with this enzyme (13-15). This suggestion appears rather unlikely in view of the proposed kinetic mechanism and because there is only one binding site for glucose-6-P. In order to test this point further we were interested in gaining information on how ATP affects glucose-6-P binding. Fig. 1 indicates that ATP is a competitive inhibitor of glucose-6-P binding and binding of these two ligands appears to be mutually exclusive. These results are in accord with the kinetic observations reported from a number of laboratories (4-7).

Although it is now known that hexokinase exists as a monomer of molecular weight of 98,000 (16), and that there is one glucose-6-P and one P_i binding site, and that these ligands exhibit mutually exclusive binding, no analogous information is available on substrate binding. Fig. 2 is a Scatchard plot (17) of glucose binding to hexokinase. The data of Fig. 2 suggest that like the product glucose-6-P, only one molecule of glucose binds per hexokinase



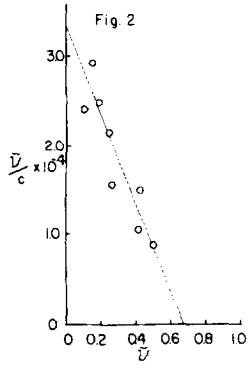


Fig. 1. A plot of reciprocal concentration of enzyme bound glucose-6-P as a function of reciprocal molar concentration of free glucose-6-P in the absence (△) and presence of 1 mM (□) and 3 mM (○) MgATP. The assay mixture contained 4.0 mM free Mg^{2+} as $MgCl_2$ (the stability constant assumed for $MgATP^{2-}$ under these conditions was 20,000 M^{-1}), 30 mM Tris-Cl, pH 7.6, 3.0 mM β-mercaptoethanol, [^{14}C] -glucose-6-P (specific activity, 272,000 c/m/nmole), MgATP where indicated, and 0.40 μM bovine brain hexokinase in a total volume of 0.25 ml. The enzyme was desalted on a Bio-Gel P-10 column in 50 mM Tris-Cl, pH 7.6 containing 5 mM β-mercaptoethanol prior to addition to the assay mixture.

Fig. 2. A Scatchard plot of glucose binding to bovine brain hexokinase. Assay mixtures contained in 0.25 ml. 30 mM Tris-Cl, pH 7.6, 3 mM β-mercaptoethanol, 4mM $MgCl_2$, [^{14}C] -glucose (specific activity, 134,000 c/m/nmole), and 0.71 μM hexokinase, desalted as described in Fig. 1. All determinations were in duplicate and presented as average values. $\bar{\nu}$ is defined as moles of ligand bound per mole enzyme and C is defined as concentration of free ligand.

polypeptide chain. The dissociation constant for E-glucose complex is 30 μM and is in agreement with the Km for glucose determined kinetically. With this hexokinase preparation the Km for ATP is 0.22 mM and is in agreement with that of Bachelard et al., (10), but considerably lower than that obtained with an enzyme prepared by extraction of mitochondria with detergents (18).

On the basis of the kinetic and binding studies, we have proposed a model for ligand binding to bovine brain hexokinase which is shown in Scheme I. In this model hexokinase is believed to exist in two rapidly interconvertible conformational states, either with (EP_i) or without (E) P_i. The rate

constants (k_1) for both forms of the enzyme are the same as are the various dissociation constants, with two exceptions; i.e., they differ for glucose-6-P binding with the two enzyme forms (i.e., $K_M >> K_L$)

We have attempted to test this model and the rate equation obtained for it, which is described as Equation 1, from kinetic experiments. Fig. 3 is

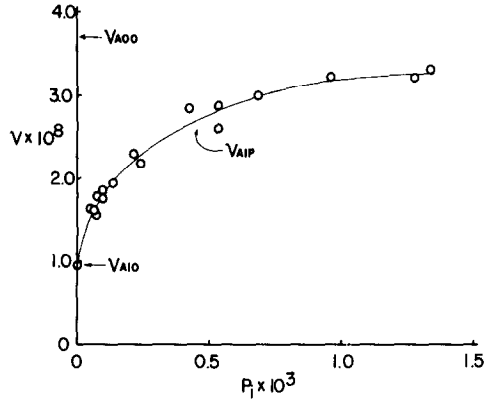


Fig. 3. A plot of initial reaction velocity (v) as a function of inorganic phosphate concentration (P_i). Reaction mixtures contained in 1.05 ml, 28 μM [^{14}C] - glucose (specific activity, 44,180 c/m/nmole), 0.25 mM ATP, 1 mM free Mg^{2+} as $Mg(NO_3)_2$, 5 μM glucose-6-P, 50 mM Tris-NO_3, pH 7.6, and 1.63×10^{-4} units of bovine brain hexokinase. The enzyme was diluted from stock into 2 mg per ml bovine serum albumin just prior to the experiment. Reactions were stopped at appropriate times to assure linearity with 0.2 ml 20 mM $AgNO_3$ containing 350 mM glucose. The [^{14}C]-glucose-6-P produced was assayed as previously reported (11). Initial velocity (v) is expressed as M glucose-6-P produced per min. V_{AIP} equals the reaction velocity in the presence of glucose-6-P, V_{AIO} equals the reaction velocity in the presence of glucose-6-P and absence of P_i, and V_{A00} equals the reaction velocity in the absence of glucose-6-P and P_i. V_{A00} was measured in a reaction mixture lacking both glucose-6-P and P_i.

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a plot of velocity versus P_i concentration in the presence of substrates and glucose-6-P for bovine brain hexokinase. Kosow et al., (7), in an attempt to explain the activation of glucose-6-P inhibited type I hexokinase, presented an empirical rate equation in which the K_i, the dissociation constant for the E-glucose-6-P complex, was modified by a function of P_i,

$f(P_i)$, which was shown (7) to be equal to $V_{AIP}(V_{AOO}-V_{AIO})/V_{AIO}(V_{AOO}-V_{AIP})$. This empirical equation, though useful, was not based on a binding or kinetic model and therefore could not explain the curvature obtained by these authors in the $f(P_i)$ versus P_i plot for hexokinase type I from Sarcoma 37.

It can be shown, given the reaction mechanism of Scheme I, that the $f(P_i)$, given by Kosow et al., (7), is equal to $\frac{K + (P_i)}{K + K_L(P_i)/K_M}$ From a plot of $1/f(P_i)$ with respect to $1/(P_i)$ it is possible to estimate the ratio of K_L to K_M and K, the dissociation constant for P_i dissociation from EP_i, E-substrate-P_i, and E-product-P_i complexes as shown in Fig. 4.

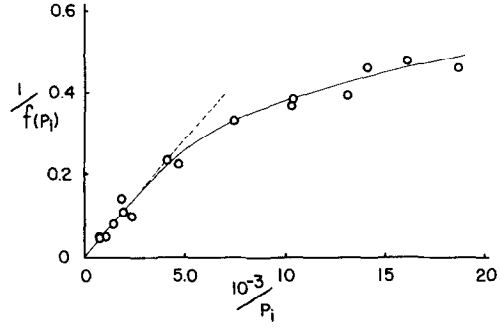

Fig. 4. A plot of reciprocal of $f(P_i)$ as a function of reciprocal molar concentration of inorganic phosphate (P_i). $f(P_i)$ is defined in the text and the velocities used to calculate $f(P_i)$ at each P_i concentration are given in Fig. 3. When P_i is high relative to K, $1/f(P_i)$ approximately equals $K/P_i + K_L/K_M$. Accordingly, K, the slope, and K_L/K_M, the intercept, are 55 μM and < 0.01, respectively.

It is clear also that at low P_i concentrations where $K_M >> K_L$, a plot of $f(P_i)$ versus (P_i) would be linear, but as P_i concentration increases, the $K_L(P_i)/K_M$ term becomes important and curvature results.

The model presented by Wilson (12), in which the enzyme exists in two states, one with bound P_i and one without P_i, but that the state with bound P_i becomes catalytically inactive, appears to be at variance with the

observation of Purich and Fromm (8) as well as Kosow et al., (7) that P_i has no effect on the Vm of the reaction.

It appears that Scheme I and rate Equation 1 are consistent with the findings of Kosow et al., (7) and those presented in this study for P_i reversal of glucose-6-P inhibition of bovine brain hexokinase. It should be pointed out that although P_i and glucose-6-P binding is mutually exclusive, these ligands probably occupy different sites. On the other hand, glucose-6-P and ATP probably compete for the same enzymic locus.

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