Kinetic Studies of the Brain Hexokinase Reaction*

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The mechanism of the hexokinase reaction has been the subject of numerous investigations in recent years (1-5). Ågren and Engström reported the incorporation of P32 from labeled adenosine triphosphate (ATP) into the phosphoserine moiety of hexokinase in the absence of substrate glucose and concluded that an enzyme-phosphate intermediate participated in the reaction (2). Gamble and Najjar, however, were unable to demonstrate a glucose-glucose 6-phosphate exchange with yeast hexokinase in the absence of nucleotide substrates (3). The latter investigators did observe labeling of hexokinase by C14 glucose in the absence of ATP (4) and suggested that an enzyme-glucose intermediate participated in the reaction. Their postulation appeared to explain adequately many of the data available from studies of the phosphotransferase. A series of recent studies appeared to be at variance with both of the above mentioned hypotheses (5).

In 1951 it was reported that glucose 6-phosphate severely inhibited mammalian hexokinase, probably noncompetitively (6). This contention was supported by investigations of Crane and Sols (7). The results of these studies implied that glucose and its phosphorylated product occupy separate enzymatic sites (6, 7). A similar view has recently been advanced regarding dehydrogenases (8); however, this explanation may not be valid (9). Hexokinase from yeast does not appear to be as susceptible to glucose 6-phosphate inhibition as the mammalian enzyme (10, 11).

Adenosine diphosphate (ADP) (12) and certain sugars (13) have been reported to be competitive inhibitors of hexokinase. Unfortunately, most of the investigations regarding inhibition were essentially preliminary.

The purpose of the present study was to investigate the mechanism of the hexokinase reaction kinetically. It will be shown that these investigations do support a mechanism in which the product of the first substrate dissociates from the enzyme before addition of the second substrate. These data appear to be consistent with the proposals that either an enzyme-phosphate or an enzyme-glucose intermediate participates in the hexokinase reaction (2, 4).

EXPERIMENTAL PROCEDURE

Materials—d-Glucose and d-mannose were obtained from Pfannstiel Laboratories, Inc. and glucose 6-phosphate, phosphoenolpyruvate, and glucose 6-phosphate dehydrogenase (type V) from Sigma Chemical Company. All other enzymes were purchased from California Corporation for Biochemical Research, and nucleotides were obtained from Pabst Laboratories.

Methods—Hexokinase was prepared from calf brain according to the procedure of Crane and Sols (14). It was found to be free of contaminating enzymes which could effect either the substrates or the products of the hexokinase system.

Velocity measurements were made in a Bausch and Lomb Spectronic 505 at 28°. Reaction mixture samples, in 0.08 M Tris-chloride buffer, pH 7.6, were kept in a water bath at 28° until used, and reactions were initiated by addition of enzyme. Initial reaction velocity was recorded as the µmoles of product produced per 9% minutes. In all experiments, except where glucose 6-phosphate inhibition was investigated, reaction mixtures contained 2 X 10-4 M TPN and glucose 6-phosphate dehydrogenase in great excess (13). Immediately before each series of kinetic experiments the hexokinase suspension containing 50% glycerol (15) was diluted with ice-cold water containing 2 mg of bovine serum albumin per ml and glucose 6-phosphate dehydrogenase. The enzyme solution was kept at 3° before use. An amount of hexokinase was used which permitted measurement of initial reaction velocities for a period of at least 12 minutes at all levels of substrates. Essentially similar results were obtained when ADP was assayed (16) after the reactions had been terminated by boiling for 1 minute at 100°.

When glucose 6-phosphate inhibition was studied, ADP formation was used as a criterion of reaction velocity. Reactions were initiated with enzyme and terminated after 9% minutes by heating for 1 minute at 100°. Samples were read in cylindrical cells of 2.5-cm light path in a Beckman DU Spectrophotometer. Control mixtures lacking glucose were analyzed similarly.

The MgCl₂ to ATP ratio in all reaction mixtures was 2:1, and when ADP inhibition was studied, MgCl₂ and ADP were added to provide a MgCl₂ to ADP ratio of 1:1; thus in all experiments to be described, the Mg to ATP ratio was maintained at 2:1. ADP was measured with lactate dehydrogenase and pyruvate kinase (16), and ATP was determined as ADP after treatment with excess glucose and hexokinase.

RESULTS

In Figs. 1 A and B and 2 A and B are shown the results obtained for the kinetics of the hexokinase reaction. These experiments were conducted in cylindrical cells of 5-cm light path, and absorbancy changes recorded were in the range 0.200 to 0.600 for 9% minutes at the lowest and highest substrate concentrations, respectively. It can be seen from the graphs that there does not appear to be any tendency for the plots to converge either at, or to the left of, the y-axis. These data are taken to mean that the two substrates, glucose and ATP, are
FIG. 1 A. Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of D-glucose. ATP concentrations were held constant at 1.97 × 10⁻⁴ M, □; 9.85 × 10⁻⁴ M, ■; 6.55 × 10⁻⁴ M, ○; and 2.62 × 10⁻⁴ M, ●. Initial reaction velocity (v) was determined as a function of D-glucose which was varied in the range 4.10 × 10⁻⁶ M to 9.85 × 10⁻⁴ M. Velocities are expressed as the molar concentration of glucose 6-phosphate formed in the reaction over a period of 23.8 minutes after addition of enzyme. Other details are as described under “Experimental Procedure.”

FIG. 1 B. Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of D-glucose. ATP concentrations were held constant at 1.88 × 10⁻³ M, □; 9.42 × 10⁻⁴ M, ○; 6.27 × 10⁻⁴ M, ■; 3.76 × 10⁻⁴ M, △; and 2.97 × 10⁻⁴ M, ●. Initial reaction velocity (v) was determined as a function of ATP which was varied in the range 2.02 × 10⁻⁴ M to 1.97 × 10⁻³ M; v is expressed as in Fig. 1. Other experimental details are given under “Experimental Procedure.”

not present on the enzyme simultaneously. If this were not true, the curves would be expected to meet at a common point. The significance of this latter possibility has been considered in detail elsewhere (17). Possibly, the simplest explanation for the kinetic data shown in Figs. 1 and 2 would be the following formulation,

$$E + A \xrightarrow{k_1} EX + C$$  \hspace{1cm} (1)
where $A$, $B$, $C$, and $D$ represent ATP, glucose, ADP, and glucose 6-phosphate, respectively.

The following steady state rate equation is derived from the above mechanism:

$$E_0 \quad v = \frac{1}{k_a} + \frac{1}{k_b(A)} + \frac{1}{k_d(B)} \left(1 + \frac{v_m}{V_m} \right),$$

where $E_0$ represents total enzyme concentration, and $v$, initial reaction velocity.

It was possible to calculate certain of the kinetic parameters with Equation 4 and the data from Figs. 1 and 2; i.e., $k_6E_0 = 2.11 \times 10^{-2}$ per minute, $k_6E_0 = 7.21 \times 10^{-4}$ m per minute, and $(1 + k_b/k_d)/k_6E_0 = 8.95$ per minute.

Simple calculation of the data from the preceding figures gives values of $2.60 \times 10^{-5}$ m and $6.00 \times 10^{-5}$ m for the apparent Michaelis constants of glucose at the lowest and highest levels of ATP, respectively. Similarly, the apparent Michaelis constants for ATP were found to be $2.92 \times 10^{-4}$ m and $1.13 \times 10^{-4}$ m at the highest and lowest concentrations of glucose, respectively. From the legends to the respective figures, it can be seen that the concentrations of substrates employed in these experiments were varied in the range below and well above the apparent Michaelis constant levels.

Equation 4, when transposed into the form suggested by Alberty (17, 18), yields the following relationship, where $V_m = k_6E_0$, $k_A = k_b/k_1$ and $k_B = (k_1 + k_d)/k_2$.

$$v = \frac{V_m}{1 + \frac{K_A}{(A)} + \frac{K_B}{(B)}}$$

From the results of the preceding figures, $K_{\text{ATP}} = 3.42 \times 10^{-4}$ m and $K_{\text{glucose}} = 6.45 \times 10^{-5}$ m.

The data thus far presented are in essential agreement with either the glucose-enzyme (4) or enzyme-phosphate (2) hypothesis. It is not possible from kinetic experiments alone to choose between these two possibilities.

In order to preclude complication of the kinetic studies by slight variations in the concentrations of MgATP$^-$, the Mg$^{++}$ to ATP ratio was maintained at 2:1. From the studies of Burton (19) and Bock (20) it would appear that Mg$^{++}$ used in these studies, neither activation nor inhibition due to cation was apparent.

Mg$^{++}$ levels of Mg$^{++}$. These experiments were carried out in cylindrical cells of 5-cm light path. It can be seen from the graphs that when the Mg$^{++}$ to ATP level was maintained between 2:1 and 3:1, there was no alteration in the apparent Michaelis constants for either ATP or glucose. Furthermore, at the levels of Mg$^{++}$ used in these studies, neither activation nor inhibition due to cation was apparent.

**d-Mannose Inhibition**—It has been suggested that d-mannose is a competitive inhibitor for d-glucose with yeast hexokinase (13). This particular hexose was chosen because it has been reported that brain hexokinase prepared by the procedure of Crane and Sols (14) is free of phosphomannoisomerase. We have been able to confirm this observation. Figs. 4 and 5 show Lineweaver-Burk type plots for mannose inhibition. It can be seen from Figs. 4 and 5 that d-mannose acts as a competitive inhibitor with respect to d-glucose and as an "uncompetitive" inhibitor with respect to ATP. If one is to assume that the brain hexokinase mechanism is similar to mechanisms suggested for certain dehydrogenases (22, 23) (at least with respect to substrate addition to the enzyme), the plot shown in Fig. 5 should be of the noncompetitive type. The data depicted in Figs. 4 and 5 are...
FIG. 4. Plot of reciprocal of initial reaction velocity (v) versus reciprocal of the molar concentration of D-glucose in the presence and absence of D-mannose. ATP was held constant at 1.18 x 10^-4 M and D-glucose varied in the range 8.00 x 10^-4 M to 6.00 x 10^-4 M. The D-mannose concentrations are shown on the figure; v is expressed as in Fig. 1. Other experimental details are given under "Experimental Procedure."

FIG. 5. Plot of reciprocal of initial reaction velocity (v) versus reciprocal of the molar concentration of ATP in the presence and absence of D-mannose. D-Glucose was held constant at 4.00 x 10^-4 M and ATP varied in the range 2.00 x 10^-4 M to 4.00 x 10^-4 M. The D-mannose concentrations are shown on the figure; v is expressed as in Fig. 1. Other experimental details are given under "Experimental Procedure."

again in basic agreement with Equation 4, except that in the presence of mannose, the equation is modified to \(EX + M \rightleftharpoons EMX\) and thus,

\[
\frac{E}{v} = \frac{1}{k_3} + \frac{1}{k_3(A)} + \frac{1}{k_3(B)} \left( \frac{1 + k_4/k_5}{1 + M/K_4} \right),
\]

where \(M\) represents mannose and \(K_4\) the dissociation constant of the mannose-EX complex. The average \(K_4\) value calculated from Fig. 4 was 3.76 x 10^-4 M and from Fig. 5, 3.45 x 10^-4 M.

It is unlikely that mannose reacts with hexokinase directly, i.e. \(E + M \rightleftharpoons EM\). This possibility would yield a rate equation that is not in harmony with the data presented in Figs. 4 and 5.

It was observed that where the experimental protocol shown in the legend to Fig. 5 was modified such that the system contained glucose at 8 \times 10^{-4} M, there was a marked increase in slope of the curves shown in Fig. 5 at mannose levels above 4 \times 10^{-4} M. The slope increase cannot be attributed to either glucose inhibition or ADP accumulation. When the level of glucose was maintained at 8.0 \times 10^{-4} M in the experiments described in Figs. 1 and 2, there was no evidence of substrate inhibition. It was observed that the level of ADP required to cause any discernible increase in the apparent Michaelis constant of ATP in the presence of ADP is approximately 2 \times 10^{-4} M (see Fig. 6). In determining initial reaction velocities, the level of ADP could never exceed 6 \times 10^{-5} M and 2.5 \times 10^{-4} M at the highest and lowest concentrations of ATP, respectively, when glucose was maintained at 8 \times 10^{-4} M. This effect then is believed to be due to mannose 6-phosphate accumulation and could represent interaction of mannose 6-phosphate with free enzyme. Derivation of an equation considering this possibility adequately accounts for this observation.

**ADP-Inhibition**—It has been suggested that ADP acted as a competitive inhibitor of ATP for the hexokinase reaction in the forward direction (12); however, we have been unable to confirm this observation. In Figs. 6 and 7 are shown double reciprocal plots with respect to substrates at different levels of ADP.

It can be seen from Fig. 6 that the addition of ADP causes a decrease in maximal velocity as well as an increase in the apparent Michaelis constant for ATP. To explain these data it is assumed that ADP reacts with the free enzyme and also with \(EY\) complex of Equation 3 above. Thus,

\[\begin{align*}
E + C & \rightleftharpoons EC; K_i = (E)/(EC) \\
EY + C & \rightleftharpoons EYC; K_{ii} = (EY)/(EYC).
\end{align*}\]

Equation 7 takes into account the effect of ADP and is as follows.

\[
\frac{E_2}{v} = \frac{1}{k_3} \left( 1 + \frac{C}{K_i} \right) + \frac{1}{k_3(A)} \left( 1 + \frac{C}{K_i} \right) + \frac{1}{k_3(B)} \left( 1 + \frac{k_4}{k_5} \right)
\]

\[
+ \frac{k_6C \left( 1 + \frac{k_7}{k_8} \right)}{k_6k_8(A)(B)}
\]

where \(C\) represents ADP. In considering Equation 7 it will be assumed that \(k_3\) is very small (see below for an explanation of this assumption).

FIG. 6. Plot of reciprocal of initial reaction velocity (v) versus reciprocal of the molar concentration of ATP in the presence and absence of ADP. D-Glucose was held constant at 8.00 x 10^-4 M and ATP varied in the range 2.00 x 10^-4 M to 3.00 x 10^-4 M. The ADP concentrations are shown on the figure; v is expressed as in Fig. 1. Other experimental details are given under "Experimental Procedure."
Fig. 7 shows results obtained when glucose is varied while ADP is held constant at three different concentrations. It can be seen that the nucleotide is apparently an uncompetitive inhibitor of the sugar. Again, Equation 7 appears to agree with the data of Fig. 7.

The possibility that the studies involving ADP inhibition might have been complicated by the presence of adenylate kinase in the enzyme preparations was excluded as follows. A reaction mixture containing $8 \times 10^{-4}$ M glucose, $2 \times 10^{-4}$ M MgCl$_2$, 0.08 M Tris buffer, pH 7.6, $1 \times 10^{-4}$ M ADP, $2 \times 10^{-4}$ M TPN, and hexokinase and glucose 6-phosphate dehydrogenase at concentrations used for the experiments shown in Figs. 6 and 7 was incubated at 28°C in a Beckman Model DU Spectrophotometer. There was no discernible alteration in absorbancy at 340 nm compared to a control mixture in which glucose had been omitted over a 1-hour period. After 1 hour, enough ATP was added to each reaction mixture to give a final ATP concentration of $1 \times 10^{-3}$ M and a dilution of other compounds of 3.9%. The addition of ATP resulted in a rapid and marked increase in absorbancy at 340 nm in the reaction mixture containing substrate glucose.

The values for $K_4$ and $K_6$, as calculated from Fig. 6 and Equation 7 are $4.20 \times 10^{-4}$ M and $1.90 \times 10^{-3}$ M, respectively. These values compare favorably, i.e. within 7%, when they are used to calculate the theoretical intercept changes on the y-axis in Fig. 7.

It had been suggested above that the rate constant $k_2$ is small. If this were not valid, and assuming the mechanism in Equations 1-3, the following term must be included in Equation 7: $k_2C(1 + k_3/k_5)(1 + k_6/k_4)/k_3k_5AB$, where $C$ represents ADP. When one measures initial reaction velocity, this term would be deleted from consideration; however, in the presence of ADP, if $k_3$ were not very small, the kinetics predicted by Equation 7 should be altered. In this case a $1/v$-glucose plot would be expected to exhibit an increased slope in the presence of ADP. That this is in fact not the case is shown in Fig. 7. It might thus be concluded that the reversal of the first step of the hexokinase reaction is relatively slow.

When the assumption is made that the rate constant $k_3$ is very small, it becomes obvious that equations of a type similar to that of Equation 4 become consistent with mechanisms where both substrates are present simultaneously on the enzyme (16). The data available for ADP inhibition are, however, in disagreement with these possibilities (22, 24).

\[ \frac{v}{E_0} = \frac{1 + k_3(D)}{k_5} + \frac{1 + k_6(B)}{k_4} + \frac{k_5k_6(D)(B)}{k_3k_4k_5(A)(B)} \]  

Equation 8

It is obvious from Equation 8 that double reciprocal plots involving ATP in the absence and presence of glucose 6-phosphate should yield a common intercept on the $1/v$ axis (see Fig. 9). On the other hand, the data of Fig. 8 appear to be at variance with Equation 8. From previously presented data, values...
The results of this report all appear to be consistent with the basic premises depicted in Equations 1 to 3. The assumptions used to account for \( \text{n-mannose and product inhibition} \) appear to be in harmony with current theory relative to enzyme inhibition. In the case of ADP inhibition, it has been suggested that the product reacts both with free enzyme and the last, or binary enzyme-product, complex. Novoa et al. (9) have reported that competitive inhibitors of heart lactic dehydrogenase may interact with the free enzyme as well as an enzyme-product complex.

As indicated earlier, it was observed that at high levels of mannose there appears to be a noncompetitive type of inhibition with glucose as a substrate when ATP is varied. This effect is that which might be expected when appreciable quantities of mannose 6-phosphate are present. These results imply that the sugar phosphate esters occupy the same enzymatic site on hexokinase. The question arises as to the validity of the kinetic equations, if as above \( k_2 \) is relatively small, and compulsory pathway mechanisms are assumed where both substrates are present on the enzyme simultaneously (18). Obviously these equations would be of the form depicted for Equation 4. However, the studies of product inhibition relative to glucose 6-phosphate and ADP are inconsistent with the equations derived by others (24, 25) for compulsory pathway mechanisms where the first step in the sequence is kinetically irreversible. Thus it would appear that the argument relative to the simultaneous presence of substrates on hexokinase is inconsistent with current theories relative to product inhibition kinetics.

**SUMMARY**

The mechanism of the brain hexokinase reaction was studied kinetically by means of product inhibition. The data obtained from these investigations appear to preclude the direct interaction of adenosine-5'-triphosphate and \( \text{n-glucose} \) in the presence of hexokinase. The results are consistent with the view that adenosine-5'-diphosphate dissociates from hexokinase before the addition of \( \text{n-glucose} \); however, the present studies do not offer direct proof of this hypothesis.

\( \text{n-Mannose acts as a competitive inhibitor of \( \text{n-glucose} \), and glucose 6-phosphate acts as a competitive inhibitor of adenosine-5'-triphosphate. Adenosine-5'-diphosphate inhibition appears to be of a more complex nature.} \)

Various rate constants were calculated from the kinetic data. The first step of the hexokinase reaction appears to be kinetically irreversible.

**Addendum**—In a personal communication, Dr. R. A. Alberty has pointed out that the data herein fit the symmetrical mechanism:

\[
\text{ATP} + \text{E} \rightleftharpoons \text{EX} \rightleftharpoons \text{E'} + \text{ADP} \text{ and } \text{Glucose} + \text{E'} \rightleftharpoons \text{EY} \rightleftharpoons \text{E} + \text{glucose 6-phosphate.}
\]

It may readily be shown that the symmetrical mechanism leads to alteration only in rate constants and not in the form of the kinetic equations of the text or the conclusions drawn from such equations.

The mechanism of yeast hexokinase has been examined kinetically in our laboratory (manuscript in preparation). The data appear to fit the pathway proposed for pyruvate kinase (26).
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