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Studies of the Enzyme Hexokinase. I. Steady State Kinetics at pH 8

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A steady state kinetic study of the transfer of a phosphate group from ATP to D-glucose as catalyzed by the enzyme hexokinase was carried out at *p*H 8 and 25.0°. The necessary divalent metal ion used was Mg^{++} and the ionic strength was 0.3 *M* in $(CH_3)_4NCl$. The results indicate that the most probable mechanism is the combination of MgATP and an enzyme–glucose complex to form a quaternary intermediate which in turn decomposes to MgADP and a dissociable enzyme–glucose-6-phosphate complex. Several rate constants and lower bounds of rate constants were calculated, and it was found that all of the bimolecular rate constants in the forward direction are greater than 10^6 M^{-1} sec.$^{-1}$ and the first order constants are greater than 700 sec.$^{-1}$. In addition the binding constant of D-glucose to hexokinase was found to be 2.5×10^3 M^{-1}.

Introduction

Although hexokinase was first crystallized a number of years ago,[1,2] surprisingly little is known about its mechanism of action. The hexokinase catalyzed transfer of a phosphate group from adenosine triphosphate (ATP) to the 6 position of various six carbon sugars is well known to require the presence of a divalent metal ion, usually Mg^{++}, but few of the details of the mechanism have been firmly established. Ågren and Engström[3] reported the isolation of an enzyme phosphate intermediate, but this finding is in conflict with recent exchange studies where labelled glucose was found not to exchange with glucose-6-phosphate in the presence of the enzyme.[4] These latter results were explained by postulating a mechanism involving a glucosyl enzyme.

Several kinetic studies have been carried out previously with rather inconclusive results. Slein, Cori and Cori[5] studied the effect on the reaction rate of varying the sugar concentration. Since they worked at constant and quite high ATP and Mg^{++} concentrations, no detailed mechanistic information was obtained. Melchoir and Melchoir[6] attempted to clarify the situation by studying the initial velocity as a function of the various ionic species present (*i.e.* ATP, MgATP, Mg^{++}); however because the role of the sugar was not taken into account, their studies could not distinguish between several plausible mechanisms. The investigation reported here will make it manifestly clear that it is not only desirable but necessary to consider simultaneous concentration variation of all of the possible reactants if a meaningful interpretation of steady state kinetics is to be made.

In this study the initial velocity of the transfer of a phosphate group from ATP to D-glucose was determined over a wide range of ATP, Mg^{++}, MgATP and glucose concentrations. At pH 8, each mole of ATP reacting is accompanied by formation of an equivalent of H^+, so that a pH stat could be used to determine these rates. In order to determine the concentration of all species present in the reaction mixture, the equilibrium quotient for MgATP formation was also determined.

The results obtained favor a mechanism proceeding through formation of a quaternary intermediate complex produced by reaction of an enzyme–glucose complex with MgATP. In addition, many of the rate constants (or lower bounds thereof) were obtained.

Experimental

Materials.—Crystalline hexokinase was prepared according to the procedure of Darrow and Colowick.[7] All hexokinase used in the kinetic experiments was crystallized three times; such enzyme is substantially free of all enzymatic impurities except for a small residual amount of ATPase activity.[8] The specific activity of the enzyme was from 200–500 Kunitz–McDonald units/mg.[1] Since the specific activity usually decreases with time, denatured hexokinase is probably always present.

The disodium salt of ATP (Pabst) was freed of sodium ion by shaking with an excess of the hydrogen ion form of Dowex 50 resin. The concentration of ATP was then determined spectrophotometrically. The pH was adjusted to the desired value with $(CH_3)_4NOH$ (Eastman Kodak Co.). This base was standardized with potassium acid phthalate in the usual manner. The $(CH_3)_4NCl$ (Eastman Kodak Co.) was recrystallized from isopropyl alcohol and dried *in vacuo*. The $MgCl_2$ and dextrose were CP grade and were used without further purification. All solutions were made from CO_2 free conductivity water.

Determination of MgATP Binding Constants.—Fifteen ml. of a solution 0.3 *M* in $(CH_3)_4NCl$, 10^{-3} *M* in ATP and 10^{-2} in $MgCl_2$ were titrated inside a thermostatted cell at 25.0° (± 0.1) C with standardized $(CH_3)_4NOH$ (approximately 0.1 *N*). A similar titration without Mg^{++} was also performed. Errors due to CO_2 absorption were prevented by working under a CO_2 free atmosphere of water saturated N_2. Titration curves were obtained using an automatic titrator manufactured by Radiometer of Copenhagen (SBR-2C and TTT 1b). Both titrations were made in triplicate.

Kinetic Measurements.—Solutions were prepared containing all possible combinations of the following total concentrations: $(ATP)_T = 5 \times 10^{-3}$ *M*, 2×10^{-3} *M*, 1×10^{-3} *M*, 5×10^{-4} *M*; $(Mg)_T$ = same as $(ATP)_T$; and dextrose = 1×10^{-3} *M*, 5×10^{-4} *M*, 2×10^{-4} *M*, 1×10^{-4} *M*. This resulted in 64 different solutions. The ionic strength was kept constant with 0.3 *M* $(CH_3)_4NCl$. Monovalent metal ions were avoided because they have been shown to inhibit the enzymatic reaction.[6] For each kinetic run, 14 ml. of solution were put into a thermostatted cell at 25.0° (± 0.1): the solution was then adjusted to approximately *p*H 8 with strong acid or base and a known amount of enzyme solution (~ 0.02 ml.) was added with a pipet. The rate of H^+ formation was then measured at *p*H 8.0 (± 0.05) using the Radiometer apparatus as a *p*H stat. Since the reaction produces H^+ and the buret injects base into the solution, any initial deviation from *p*H 8 is compensated for quite quickly. The volume of base used was always less than 0.2 ml. The glass electrode was soaked in a solution containing

(1) M. Kunitz and M. R. McDonald, *J. Gen. Phys.*, **29**, 393 (1946).

(2) L. Berger, M. Slein, S. P. Colowick and C. F. Cori, *ibid.*, **29**, 379 (1946).

(3) G. Ågren and L. Engström, *Acta Chem. Scand.*, **10**, 489 (1956).

(4) V. A. Najjar and E. E. McKay, *Federation Proc.*, **17**, 1141 (1958).

(5) M. W. Slein, G. T. Cori and C. F. Cori, *J. Biol. Chem.*, **186**, 763 (1950).

(6) N. C. Melchoir and J. B. Melchoir, *ibid.*, **231**, 609 (1958).

(7) R. A. Darrow and S. P. Colowick, in "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. V, Academic Press, Inc., New York, N. Y., 1961, p. 226.

(8) K. A. Trayser and S. P. Colowick, *Arch. Biochem. Biophys.*, **94**, 161 (1961).

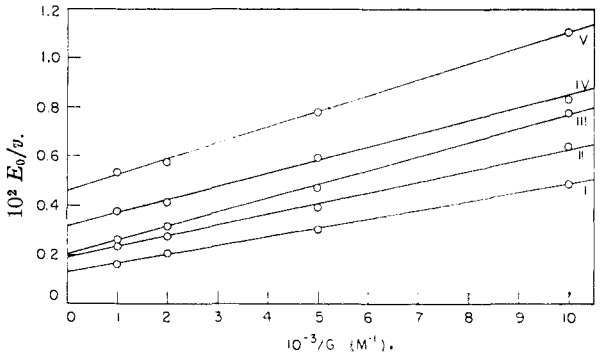

Fig. 1.—Representative plots of $(E_0)/v$ *versus* $1/(G)$ at various total concentrations of ATP and Mg: I. $(ATP)_T = 2 \times 10^{-3}$ *M*, $(Mg)_T = 5 \times 10^{-3}$ *M*; II. $(ATP)_T = (Mg)_T = 1 \times 10^{-3}$ *M*; III. $(ATP)_T = 5 \times 10^{-4}$ *M*, $(Mg)_T = 1 \times 10^{-3}$ *M*; IV. $(ATP)_T = 1 \times 10^{-3}$ *M*, $(Mg)_T = 5 \times 10^{-4}$ *M*; V. $(ATP)_T = 5 \times 10^{-3}$ *M*, $(Mg)_T = 5 \times 10^{-4}$ *M*.

enzyme prior to use and the calomel electrode tip was covered with a thin film of silicone grease to minimize the flow of saturated KCl solution. Again a CO_2 free atmosphere of water saturated nitrogen was employed. The titrant was standardized $(CH_3)_4NOH$ (approximately 0.01 *N*). Since each equivalent of H^+ formed corresponds to a mole of ATP reacting, the initial slope of the ml of titrant added *vs.* time curve is a direct measure of the initial reaction velocity. Blank corrections for CO_2 absorption, ATPase activity and the buffer capacity of the system were negligible.

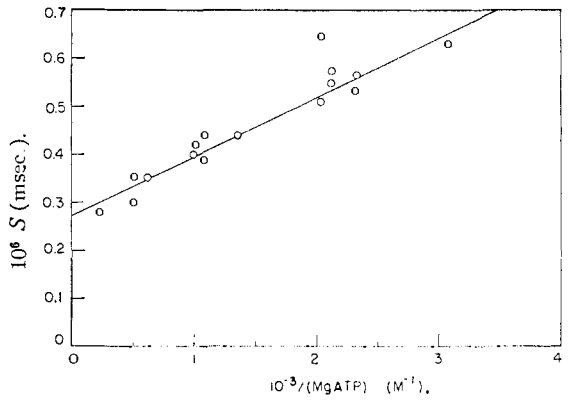

Fig. 2.—The slopes of $(E_0)/v - 1/(G)$ plots (*e.g.* Fig. 1) *versus* $1/(MgATP)$.

The enzyme solution was prepared by centrifuging down the crystals, decanting the liquid and dissolving them in an appropriate amount of ice cold water. The concentration was adjusted to give conveniently measurable initial velocities ($\sim 10^{-9}$ *M*). Although the enzyme was kept in an ice bath, it was found to slowly lose activity. This effect was corrected for by referring all rates to that of a standard solution [$(ATP)_T$ = (Dextrose) = 10^{-3} *M*, $(Mg^{++})_T = 5 \times 10^{-3}$ *M*] whose initial velocity was determined every hour. The decay of activity seldom amounted to more than 10% a day. The actual concentration of pure enzyme in the standard solution was found by using the Kunitz–McDonald assay together with their data for the specific activity of the pure enzyme and a molecular weight of 96,600.[1] This assumes one active site per molecule. The release of 1×10^{-7} *M*/sec. of H^+ in the standard solution was found to correspond to an enzyme concentration of 2.06×10^{-10} *M*.

Since this work was completed Trayser and Colowick[9] have shown that crystalline hexokinase can be resolved into

(9) K. Trayser and S. P. Colowick, *Arch. Biochem. Biophys.*, **94**, 177 (1961).

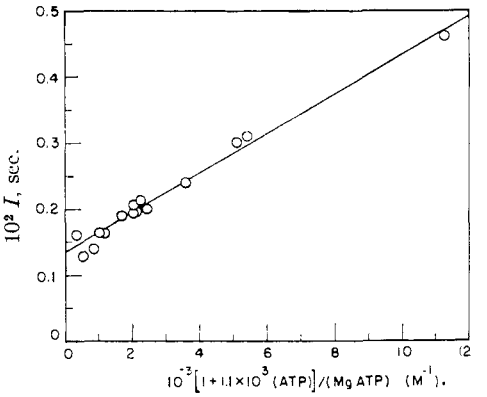

Fig. 3.—The intercepts of $(E_0)/v - 1/(G)$ plots (*e.g.* Fig. 1) *versus* $[1 + 1.1 \times 10^3(ATP)]/(MgATP)$.

several isozymes. This will not affect the conclusions presented here providing all of the isozymes utilize the same mechanism.

Results

Equilibrium Quotients.—The acid dissociation constants of ATP and the Mg^{++} binding constant were calculated from the titration data using the procedure of Martell and Schwarzenbach.[10] The results at 25.0 (± 0.1) and an ionic strength of 0.3 *M* $[(CH_3)_4NCl]$ are as follows:

$$K_{AH_2} = \frac{(ATPH_2^{-2})}{a_H(ATPH^{-3})} = 2.24 \times 10^4\ M^{-1}$$

$$K_{AH} = \frac{(ATPH^{-3})}{a_H(ATP^{-4})} = 8.52 \times 10^5\ M^{-1}$$

$$K_{AMH} = \frac{(MgATPH^{-1})}{(Mg^{++})(ATPH^{-3})} = 1.26 \times 10^2\ M^{-1}$$

$$K_{AM} = \frac{(MgATP^{-2})}{(Mg^{++})(ATP^{-4})} = 1.17 \times 10^4\ M^{-1}$$

Here a_H designates the hydrogen ion activity as measured by the glass electrode, while all other species are given in concentration units. These results are in reasonable agreement with those in the literature[6,10,11] and can be used to calculate the concentrations of the ionic species in solution.

Kinetics.—Since the kinetic data are so numerous, it is not feasible to tabulate all of the results here; however, a summation of the 64 initial velocities and the concentrations of the individual species in solution are available.[12] The concentration of free ATP was varied from 9.21×10^{-5} *M* to 4.12×10^{-3} *M*, that of MgATP from 3.26×10^{-4} *M* to 4.36×10^{-3} *M* and that of free Mg^{++} from 1.01×10^{-5} *M* to 4.51×10^{-3} *M*. (It should be mentioned that some of these initial velocities were measured under identical conditions

(10) A. E. Martell and G. Schwarzenbach, *Helv. Chim. Acta.*, **39**, 653 (1956).

(11) R. M. Smith and R. A. Alberty, *J. Phys. Chem.*, **60**, 180 (1956).

(12) These data have been deposited as Document number 6949 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting $1.25 for photoprints, or $1.25 for 35 mm. microfilm. Advance payment is required. Make checks or money order payable to: Chief, Photoduplication Service, Library of Congress.

to those employed by Melchoir and Melchoir.[6] In these cases agreement within experimental error (5 to 10%) was obtained if an appropriate normalization was used.)

The rate law found to fit the data best will now be given and the adequacy of this representation will be demonstrated. The ratio of the total enzyme concentration to the initial velocity, $(E_0)/v$, can be represented within experimental error by the following equation:

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)}[1 + K_i(ATP)] + \frac{\phi_4^*}{(G)(MgATP)}$$

where G represents D-glucose and $\phi_1 = 1.34 \times 10^{-3}$ sec., $\phi_2 = 2.71 \times 10^{-7} M$ sec., $\phi_3 = 3.02 \times 10^{-7} M$ sec., $\phi_4^* = 1.23 \times 10^{-10} M^2$ sec., and $K_i = 1.1 \times 10^3 M^{-1}$. The estimated *maximum* error in the initial velocities is ± 5%, that in the ϕ's is ± 15% and that in K_i is ± 20%. This assumes an error of less than ± 5% in (E_0); actually the absolute error in determining (E_0) is larger due to the difficulty of obtaining great accuracy with the Kunitz–McDonald assay. However, since all initial velocities were referred to a standard within ± 5%, the relative values of the ϕ's are correct within the error limits cited.

The above rate equation predicts that at constant total concentrations of ATP and Mg^{++}, $(E_0)/v$ is a linear function of 1/(G) with an intercept equal to $\phi_1 + \phi_3[1 + K_i(ATP)]/(MgATP)$ and a slope equal to $\phi_2 + \phi_4^*/(MgATP)$. Figure 1 shows some typical plots of $(E_0)/v$ *versus* 1/(G) at various total concentrations of ATP and Mg^{++}. The slope of this plot should be a linear function of 1/(MgATP); Fig. 2 shows such a plot where the line was determined by the method of least squares. Only one point deviates appreciably from the line, *i.e.* greater than about 10%; this is at the highest concentration of free ATP and probably indicates the onset of some type of noncompetitive ATP inhibition. Therefore, this point was not included in the least squares treatment. Figure 3 shows a plot of the intercept *versus* $[1 + K_i(ATP)]/(MgATP)$; again the equation of the line was calculated using the method of least squares. The constant K_i was determined by using the value which minimized the average deviation of the experimental points from the line. One point was omitted on the statistical basis that its deviation from the line (~ 35%) is greater than 4 times the average deviation. Including this point in the least square calculations changed ϕ_1 and ϕ_3 by less than 2%. From these figures, it can be seen that the proposed rate equation gives a good description of a large amount of data.

Discussion

Eight mechanisms were examined to see if their initial velocities were consistent with the experimental results. In brief, these mechanisms are (1) formation of an enzyme–Mg complex followed by reaction with ATP to give ADP and an enzyme–Mg–phosphate intermediate which in turn reacts with glucose; (2) formation of an enzyme–phosphate intermediate and MgADP by a reaction between MgATP and enzyme, this step again being followed by reaction with glucose; formation of a quaternary Mg–ATP–enzyme–glucose complex preceded in order by the complexes (3) enzyme–Mg–ATP and enzyme–Mg, or (4) enzyme–Mg–ATP and enzyme–ATP, or (5) enzyme–ATP–glucose and enzyme–ATP; or (6) enzyme–ATP–glucose and enzyme–glucose; and formation of a quaternary complex by (7) the reaction of MgATP with enzyme, the resulting intermediate combining with glucose, or (8) the reaction of MgATP with an enzyme-glucose complex. The reverse mechanisms are assumed to be symmetrical with the forward, *i.e.*, merely the substitution of ADP for ATP and glucose-6-phosphate for glucose. These mechanisms can be represented by the reaction schemes

1. $E + Mg \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EMg$

$$EMg + ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'Mg + ADP$$

$$E'Mg + G \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} Y_1 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EMg + G6P$$

2. $ATP + Mg \rightleftharpoons MgATP$

$$MgATP + E \rightleftharpoons X_1 \rightleftharpoons E' + MgADP$$

$$E' + G \rightleftharpoons Y_1 \rightleftharpoons E + G6P$$

$$MgADP \rightleftharpoons ADP + Mg$$

3. $E + Mg \rightleftharpoons EMg$

$$EMg + ATP \rightleftharpoons EMgATP$$

$$EMgATP + G \rightleftharpoons X_1 \rightleftharpoons EMgADP + G6P$$

$$EMgADP \rightleftharpoons EMg + ADP$$

4. $E + ATP \rightleftharpoons EATP$

$$EATP + Mg \rightleftharpoons EATPMg$$

$$EATPMg + G \rightleftharpoons X_1 \rightleftharpoons EADPMg + G6P$$

$$EADPMg \rightleftharpoons EADP + Mg$$

$$EADP \rightleftharpoons E + ADP$$

5. $E + ATP \rightleftharpoons EATP$

$$EATP + G \rightleftharpoons EATPG$$

$$EATPG + Mg \rightleftharpoons X_1 \rightleftharpoons EADPG6P + Mg$$

$$EADPG6P \rightleftharpoons EADP + G6P$$

$$EADP \rightleftharpoons E + ADP$$

6. Same as 5 except ATP and ADP are interchanged with G and G6P respectively

7. $ATP + Mg \rightleftarrows MgATP$

$MgATP + E \rightleftarrows EMgATP$

$EMgATP + G \rightleftarrows X_1 \rightleftarrows EMgADP + G6P$

$EMgADP \rightleftarrows MgADP + E$

$MgADP \rightleftarrows ADP + Mg$

8. $ATP + Mg \rightleftarrows MgATP$

$E + G \rightleftarrows EG$

$MgATP + EG \rightleftarrows X_1 \rightleftarrows EG6P + MgADP$

$EG6P \rightleftarrows E + G6P$

$MgADP \rightleftarrows ADP + Mg$

Here E represents the free enzyme, X_1 and Y_1 are enzyme intermediates, G6P is glucose-6-phosphate, and other symbols have their obvious meanings. Rate constants can be assigned to all the mechanisms in a manner similar to that employed in mechanism 1. The initial velocities can be written in terms of the rate constants[13] and initial concentrations or alternatively the familiar maximum velocities, Michaelis constants and equilibrium constants[14] may be used. Since this latter notation is rather combersome for such complicated mechanisms, the initial steady state velocities will be expressed in terms of experimentally determinable constants designated by ϕ's. These constants can be related to the rate constants of a given mechanism. Table I contains a tabulation giving the expressions for these steady state parameters in terms of the rate constants for each mechanism. The ratio of the total enzyme concentration, (E_0), to the initial velocities, v for these eight mechanism can be written as

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(ATP)}\left(1 + \frac{\phi_4}{(Mg)}\right) \quad (1)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)} \quad (2)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(ATP)}\left(1 + \frac{\phi_4}{(Mg)}\right) + \frac{\phi_2\phi_5}{(ATP)(G)}\left(1 + \frac{\phi_4}{(Mg)}\right) \quad (3)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)}\left(1 + \frac{\phi_4}{(Mg)}\right) + \frac{\phi_3}{(ATP)}\left(1 + \frac{\phi_5}{(Mg)}\right) + \phi_6(Mg) + \frac{\phi_7}{(Mg)} + \frac{\phi_2\phi_8}{(ATP)(G)(Mg)} \quad (4)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(ATP)} + \frac{\phi_4}{(Mg)}\left(1 + \frac{\phi_5}{(G)}\right) + \phi_6(Mg) + \frac{\phi_7}{(ATP)(G)} + \frac{\phi_4\phi_8}{(ATP)(G)(Mg)} \quad (5)$$

Same as 5 except ATP and ADP are interchanged with G and G6P, respectively (6)

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)} + \frac{\phi_2\phi_4}{(G)(MgATP)} \quad (7)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)} + \frac{\phi_3\phi_4}{(G)(MgATP)} \quad (8)$$

In mechanisms 2, 7 and 8, the first and last steps have been assumed rapid compared to the over-all reaction; this is in accord with recent experimental results.[15,16] If equations 1–8 are examined carefully, it will be seen that *except for mechanisms 7 and 8 which have initial velocities that are identical functions of the various concentrations*, all of the mechanisms are (in principle) experimentally distinguishable. The data obtained do not fit the first six mechanisms in even a qualitative manner. On the other hand, the initial velocity functions for mechanisms 7 and 8 are identical to the experimental rate law *if* $K_1 = 0$. Thus the data at low concentrations of free

TABLE I

DEFINITIONS OF STEADY STATE KINETIC CONSTANTS

Mechanism	ϕ_1	ϕ_2	ϕ_3	ϕ_4	ϕ_5	ϕ_6	ϕ_7	ϕ_8
1	$\frac{1}{k_3} + \frac{1}{k_5}$	$\frac{k_{-4} + k_5}{k_4k_5}$	$\frac{k_{-2} + k_3}{k_2k_3}$	$\frac{k_{-1}}{k_1}$	..	..	..	....
2	$\frac{1}{k_3} + \frac{1}{k_5}$	$\frac{k_{-4} + k_5}{k_4k_5}$	$\frac{(k_{-2} + k_3)}{k_2k_3}$	..	..	..	..	....
3	$\frac{1}{k_4} + \frac{1}{k_5}$	$\frac{k_{-3} + k_4}{k_3k_4}$	$\frac{1}{k_2}$	$\frac{k_{-1}}{k_1}$	$\frac{k_{-2}}{k_2}$	..	..	....
4	$\frac{1}{k_4} + \frac{1}{k_5} + \frac{1}{k_6}$	$\frac{k_{-3} + k_4}{k_3k_4}$	$\frac{1}{k_1}$	$\frac{k_{-2}}{k_2}$	$\frac{k_{-1}}{k_2}$	$\frac{k_{-5}}{k_5k_6}$	$\frac{1}{k_2}$	$\frac{k_{-1}k_{-2}}{k_1k_2}$
5 and 6	$\frac{1}{k_4} + \frac{1}{k_5} + \frac{1}{k_6} + \frac{k_{-3}k_{-4}}{k_3k_4k_5}$	$\frac{1}{k_2}\left(1 + \frac{k_{-2}k_{-3}k_{-4}}{k_3k_4k_5}\right)$	$\frac{1}{k_1}$	$\frac{k_{-3} + k_1}{k_3k_4}$	$\frac{k_{-2}}{k_2}$	$\frac{k_{-4}}{k_4k_5}$	$\frac{k_{-1}}{k_1k_2}$	$\frac{k_{-1}k_{-2}}{k_1k_2}$
7	$\frac{1}{k_4} + \frac{1}{k_5}$	$\frac{k_{-3} + k_{-4}}{k_3k_4}$	$\frac{1}{k_2}$	$\frac{k_{-2}}{k_2}$	..	..	..	....
8	$\frac{1}{k_4} + \frac{1}{k_5}$	$\frac{1}{k_2}$	$\frac{(k_{-3} + k_4)}{k_3k_4}$	$\frac{k_{-2}}{k_2}$	..	..	..	....

(13) E. L. King and C. Altman, *J. Phys. Chem.*, **60**, 1375 (1956).

(14) R. A. Alberty, *Adv. in Enzymology*, **17**, 1 (1956).

(15) H. Diebler, M. Eigen and G. G. Hammes, *Z. Naturforsch.*, **156**, 554 (1960).

(16) M. Eigen and G. G. Hammes, *J. Am. Chem. Soc.*, **82**, 5951 (1960); **83**, 2786 (1961).

ATP are well represented by the rate laws for these two mechanisms. The most reasonable explanation of the K_i(ATP) term is that MgATP is the true substrate, while ATP inhibits the reaction. In fact, this inhibition permits a distinction to be made between mechanisms 7 and 8.

The competitive inhibition in these two mechanisms can be written as

7. $E + ATP \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} EATP$ (inactive)

8. $EG + ATP \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} EGATP$ (inactive)

The steady state initial velocities for mechanisms 7 and 8, respectively, are now

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)}[1 + K_i(ATP)] + \frac{\phi_2\phi_4}{(MgATP)(G)}[1 + K_i(ATP)] \quad (9)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)}[1 + K_i(ATP)] + \frac{\phi_3\phi_4}{(MgATP)(G)} \quad (10)$$

where $K_i = k_7/k_{-7}$. Equation 9 fits the data very poorly, while equation 10 is identical with the experimentally determined equation given in the results section. Inhibition by combination of of ATP with X_1, EMgATP (in 7) and E (in 8) were also considered, but the resulting rate laws were inconsistent with the experimental results. Thus the conclusion of this study is that only mechanism 8 (of those considered) explains all of the data in a satisfactory manner. The formation of an enzyme–glucose complex is postulated, but it is *not* "undissociable" as proposed in the mechanism of Najjar and McKay[4] on the basis of their exchange data. In fact, the mechanism proposed here is consistent with their results, but their mechanism is *not* consistent with the steady state kinetic data. An enzyme–phosphate intermediate cannot be ruled out *if* the phosphate transfer occurs only when both substrates are bound to the enzyme. Although this mechanism may not sound attractive, it certainly is possible that both substrates are necessary in order for the enzyme to have the correct conformation for phosphate transfer. In this same vein, a very attractive hypothesis is that the binding of glucose by the enzyme changes the enzyme conformation sufficiently to enhance greatly the binding of MgATP occuring in the next step. This matter is being investigated further.

Only two of the rate constants can be calculated exactly from the steady state data presented; however, lower bounds can be found for many of the rate constants using a procedure similar to that employed by Peller and Alberty.[17] The results obtained are

$k_2 = 1/\phi_2 = 3.7 \times 10^6\ M^{-1}$ sec.$^{-1}$ $\quad k_4 > 1/\phi_1 = 750$ sec.$^{-1}$

$k_{-2} = \phi_4/\phi_2 = 1.5 \times 10^3$ sec.$^{-1}$ $\quad k_5 > 1/\phi_1 = 750$ sec.$^{-1}$

$k_3 > 1/\phi_3 = 3.3 \times 10^6\ M^{-1}$ sec.$^{-1}$

Due to the difficulty of obtaining the absolute value of (E_0) accurately, the rate constants are probably certain to only ± 40%. From previous work,[13,14] it is also known that at 25° and in 0.1 M KNO_3, $k_1 = 1.2 \times 10^7\ M^{-1}$ sec.$^{-1}$, $k_{-1} = 1.2 \times 10^3$ sec.$^{-1}$, $k_6 = 3 \times 10^6\ M^{-1}$ sec.$^{-1}$ and $k_{-6} = 2.5 \times 10^3$ sec^{-1}. The association constant for the enzyme-glucose complex is $2.5 \times 10^3\ M^{-1}$ (± 25%). This is about 60% smaller than the value found by Trayser and Colowick[18] by an indirect method. The rate constants will be discussed more fully in the next paper of this series.[19]

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Studies of the Enzyme Hexokinase. II. Kinetic Inhibition by Products

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The rate of transfer of a phosphate group from ATP to D-glucose is retarded by the presence of initial concentrations of the products, ADP and glucose-6-phosphate. This inhibition is in quantitative agreement with the previously proposed mechanism of action for the enzyme hexokinase. From these studies, the binding constants for glucose-6-phosphate and free enzyme and for MgATP and an enzyme–glucose complex were found to be $1.1 \times 10^2\ M^{-1}$ and $6.2 \times 10^3\ M^{-1}$, respectively. The latter constant is only about six times larger than the binding constant for the enzyme–glucose complex and free ATP. ($1.1 \times 10^3\ M^{-1}$). This suggests that Mg is not a very important factor for binding of the substrate to the enzyme; instead the primary role of Mg is probably to aid in the bond breaking step in the reaction. Combining all of the kinetic data available with the equilibrium constant for the over-all reaction permits all twelve of the rate constants in the mechanism to be determined.

Introduction

The previous paper in this series[1] proposed the following mechanism for the hexokinase catalyzed transfer of a phosphate group from adenosine triphosphate (ATP) to D-glucose

(1) G. G. Hammes and D. Kochavi, *J. Am. Chem. Soc.*, **84**, 2069 (1962).