# KINETIC STUDIES OF SOLUBILIZED BRAIN HEXOKINASE WITH D-FRUCTOSE AS A SUBSTRATE*

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The mechanism of action of "kinase" type phosphotransferase enzyme systems appears to be sequential with only one exception. It is believed that yeast hexokinase (Fromm and Zewe, 1962a; Fromm, et al., 1964; Zewe, et al., 1964), pyruvate kinase (Reynard, et al., 1961), creatine kinase (Morrison and Cleland, 1966), and galactokinase (Gulbinsky and Cleland, 1968) require that all substrates be present on the enzyme before product formation can occur. On the other hand, initial velocity studies have suggested that both particulate (Fromm and Zewe, 1962b) and solubilized (Copley and Fromm, 1967) brain hexokinase exhibit a ping-pong mechanism of enzyme and substrate interaction. Two types of mechanisms have been proposed for brain hexokinase (Fromm and Zewe, 1962b; Copley and Fromm, 1967).

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One of these involves participation of an enzyme-phosphate intermediate and the other an enzyme-glucose or enzyme-glucose-6-P intermediate, e.g.:

$$E + ATP = E - P + ADP$$
$$E - P + Glucose = E + Glucose\text{-}6\text{-}P \quad (1)$$

$$E\text{-}Glucose + ATP = E\text{-}Glucose\text{-}6\text{-}P + ADP$$
$$E\text{-}Glucose\text{-}6\text{-}P + Glucose = E\text{-}Glucose + Glucose\text{-}6\text{-}P \quad (2)$$

Although we have been able to demonstrate reversal of the brain hexokinase reaction at high $Mg^{2+}$ concentrations (0.05 M), we have not been able to discern a glucose - glucose-6-P exchange reaction in the absence of nucleotide substrates with the solubilized enzyme. Furthermore, we have not been able to label hexokinase with $^{32}P$ when the enzyme is incubated with $\gamma$ labeled $^{32}P$-ATP followed either by exhaustive dialysis or passage through Biogel P2. Similarly, it has not been possible for us to show an incorporation of either $^{14}C$-glucose or $^{14}C$-glucose-6-P into hexokinase when these substrates are incubated with the enzyme in the absence of ATP or ADP and then subjected to dialysis or Biogel P2 treatment. Although an ADP-ATP exchange reaction has been demonstrated in the absence of sugar substrates, it is not thought to be mediated by hexokinase, i.e., 0.1 M N-acetylglucoseamine inhibits the hexokinase reaction completely, however, it has no effect on the ADP-ATP exchange.

Additional kinetic studies were therefore undertaken in an attempt to reconcile the conclusions alluded to from initial rate experiments which suggested a ping-pong mechanism for hexokinase (Fromm and Zewe, 1962b; Copley and Fromm, 1967) and the negative findings observed for the exchange reactions.

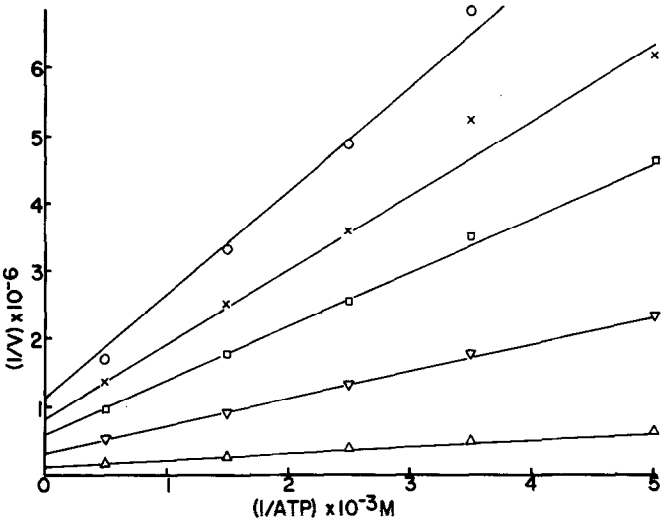


Figure 1: Plot of reciprocal of initial velocity (v) versus reciprocal of molar concentration of ATP. D-fructose concentrations were held constant at 1.82 (Δ), 0.364 (▽), 0.182 (□), 0.121 (X), and 0.091 mM (O). v was determined as a function of ATP concentration which was varied in the concentration range 0.2 to 2.0 mM. Velocities are expressed as the molar concentration of fructose-6-P formed in the reaction mixture over a period of 1 min. after addition of enzyme at 28°. Initial velocities were determined in a Cary 15 spectrophotometer (0-0.1 slide wire) in 3.3 ml reaction mixtures using a solubilized preparation of brain hexokinase prepared by the method of Schwartz and Basford (1967). The reaction mixture samples contained 0.12 M Tris-chloride buffer, pH 7.6, 50 μM TPN, 0.8 units phosphoglucoisomerase (Calbiochem.), 1.0 unit glucose-6-P dehydrogenase (Calbiochem.) and $MgSO_4$. The concentration of the $Mg^{2+}$ was adjusted so as to maintain the free $Mg^{2+}$ at 1 mM (Copley and Fromm, 1967). Fructose, which was recrystallized twice from methanol, was assayed enzymatically with excess ATP, hexokinase, phosphoglucoisomerase, and glucose-6-P dehydrogenase. ATP was assayed enzymatically with excess glucose, hexokinase, and glucose-6-P dehydrogenase.

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In Figs. 1 and 2 are shown initial rate data for solubilized brain hexokinase when the substrates are D-fructose and ATP. Experimental details are listed in the legend to the graphs. These experiments were repeated using a coupled ADP assay (Copley and Fromm, 1967) and the results were similar to those shown here. The significant feature of the data is the obvious convergence of the curves to the left of the 1/v axis.

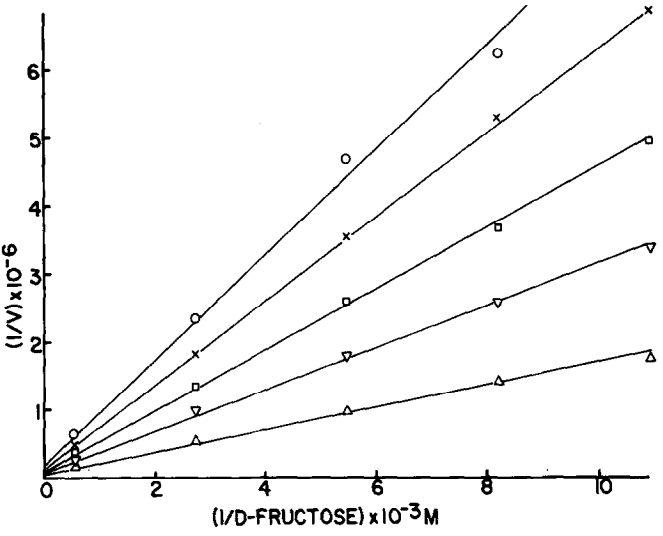


Figure 2: Plot of reciprocal of initial velocity (v) versus reciprocal of molar concentration of D-fructose. ATP concentrations were held constant at 2.0 (Δ), 0.67 (▽), 0.40 (□), 0.29 (X), and 0.20 mM (O). v was determined as a function of D-fructose concentration which was varied in the concentration range 0.091 to 1.82 mM. Other experimental details are given in legend to Fig. 1.

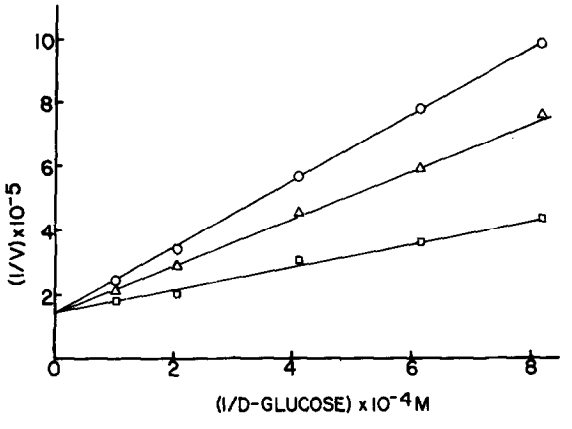


Figure 3: Plot of reciprocal of initial velocity (v) versus reciprocal of molar concentration of D-glucose in the presence and absence of D-fructose. The concentration of ATP was maintained at 1.35 mM and D-glucose was varied in the concentration range from 96 μM to 12 μM. The concentrations of D-fructose were none (□), 2.0 mM (Δ), and 3.9 mM (O). Other experimental details are given in legend to Fig. 1 except that phosphoglucoisomerase was omitted from the reaction mixtures.

These results appear to rule out a ping-pong mechanism for this enzyme. In order to show that both glucose and fructose react at the same site on hexokinase, a study of competitive inhibition, as indicated in Fig. 3, was undertaken. It is clear from the data that the two sugar substrates compete for the same locus on the enzyme.

There seems little doubt from the experiments presented here that the ping-pong mechanism suggested for brain hexokinase (Fromm and Zewe, 1962a; Fromm, et al., 1964; Zewe, et al., 1964) is incorrect. In order to rationalize the former kinetic data with a sequential mechanism, it is necessary to realize that the rate equation (Dalziel, 1957) for a sequential mechanism contains a $\phi_{12}$ term while this term is absent in the case of ping-pong mechanisms, i.e.,

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{(ATP)} + \frac{\phi_2}{(Fructose)} + \frac{\phi_{12}}{(ATP)\ (Fructose)} \qquad (3)$$

It appears then for brain hexokinase that $\phi_{12}$ is much smaller than the other terms of the rate equation when glucose is the substrate rather than fructose.

The other data which appear to support a ping-pong mechanism for the brain enzyme involve initial rate studies of the hexokinase reaction when glucose and ATP are substrates and AMP and ADP are inhibitors (Copley and Fromm, 1967). If these nucleotides can bind to the enzyme at the ATP site and another inhibitory site on the enzyme, the results would be in agreement with a sequential mechanism. This possibility has already been considered in detail (Copley and Fromm, 1967).

The findings of the present report indicate that the mechanism of action of solubilized brain hexokinase is

sequential and not ping-pong as previously supposed. It is possible, however, to rationalize the former kinetic results (Fromm and Zewe, 1962b; Copley and Fromm, 1967) with a sequential mechanism by making some relatively simple assumptions. The validity of these assumptions is currently being investigated.

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