Initial-rate studies of a thermophilic glucokinase from *Bacillus stearothermophilus*

Haruo ISHIKAWA,* Takekazu MAEDA and Haruo HIKITA

Department of Chemical Engineering, University of Osaka Prefecture, 804 Mozu-Umemachi 4-cho, Sakai, Osaka 591, Japan

The initial rates of phosphorylation of glucose catalysed by glucokinase from *Bacillus stearothermophilus* were measured over a wide range of glucose, MgATP^{2-} , MgADP^- and glucose 6-phosphate concentrations. The results of the effects of the inhibitors on the initial rates suggest that the reaction mechanism is essentially the ordered Bi Bi, in which glucose adds to the enzyme before MgATP^{2-} and glucose 6-phosphate is released from the enzyme after the dissociation of MgADP^- , and also suggest that the final step in which glucose 6-phosphate is released is irreversible. For many reaction schemes, the rate equations were derived on the basis of the pseudo-steady-state assumption and were used to correlate the experimental rate data. From this result, we concluded that the reaction obeys the ordered mechanism accompanied by the formation of a non-productive ternary complex, glucose– MgADP^- –enzyme. By using the experimental Dalziel coefficients ϕ_i , some kinetic parameters were evaluated. The enzyme was characterized by the thermal stability and the low Michaelis constant, the values of which were 54 μM for glucose and 32 μM for MgATP^{2-} .

INTRODUCTION

Glucokinase (EC 2.7.1.2) is one of the hexokinases, which catalyse phosphorylation of hexose to hexose 6-phosphate, but is very specific for glucose. Many glucokinases have been isolated from various sources, such as rat liver (Parry & Walker, 1966), *Aerobacter aerogenes* (Kamel *et al.*, 1966), *Bacillus stearothermophilus* (Hengartner & Zuber, 1973), *Zymomonas mobilis* (Scopes *et al.*, 1985), *Streptococcus mutans* (Porter *et al.*, 1982), *Brevibacterium fuscum* (Saito, 1965) and *Entamoeba histolytica* (Reeves *et al.*, 1967).

Hexokinases and glucokinases are relatively labile. However, the glucokinase from *B. stearothermophilus* is thermostable and thus is a suitable enzyme for structural and functional studies. Furthermore, it appears to be especially suitable for use as an analytical enzyme or an industrial catalyst.

Whereas the mechanism of the glucose phosphorylation by glucokinase from rat liver (Parry & Walker, 1967; Storer & Cornish-Bowden, 1976b, 1977; Gregoriou *et al.*, 1981) has been investigated intensively, there is almost no information about the kinetics and the mechanism of glucokinase from *B. stearothermophilus*. In the present work we measured the initial reaction rates of the phosphorylation of glucose catalysed by glucokinase from *B. stearothermophilus*. By analysing the results, we investigated the reaction mechanism and evaluated the kinetic constants.

MATERIALS AND METHODS

Materials

The disodium salts of ATP and ADP, NADH, NADP^+ , glucose 6-phosphate, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and glucose oxidase were obtained from Oriental Yeast Co., Osaka, Japan. The glucokinase from *B. stearothermophilus*, in which the specific activity is about 350 units/mg of protein at pH 9.0 and 303 K (Enzymes, p. 23, brochure published by Unitika), was supplied by Unitika, Osaka, Japan. Peroxidase, *o*-dianisidine and other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Solutions of reagents were made up in 0.1 M-triethanolamine/0.1 M-HCl adjusted to pH 7.5 with aq. KOH.

Enzyme assays

The method used in most of the kinetic experiments was a coupled assay (Slein *et al.*, 1950; Storer & Cornish-Bowden, 1974) with glucose-6-phosphate dehydrogenase as the coupling enzyme. The formation of glucose 6-phosphate was followed indirectly by measuring enzymically the amount of NADPH formed at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in a Shimadzu-120-02 spectrophotometer. The reaction was carried out at 310.2 K in a total volume of 3 ml of 0.1 M-triethanolamine buffer, pH 7.5, containing 0.05–10 mM-glucose, 0.005–1.0 mM-ATP, 0–1.0 mM-ADP, 10 mM- MgCl_2 , 1.0 mM- NADP^+ , 1 unit of glucose-6-phosphate dehydrogenase/ml and 0.125 μg of glucokinase/ml. The experimental time courses of the absorbance change were analysed by a non-linear-regression procedure to obtain the initial reaction rates.

Another coupled assay (Parry & Walker, 1966; Storer & Cornish-Bowden, 1976b) was also used, for the study of the inhibition by glucose 6-phosphate. In this case the rate was measured by coupling the production of ADP to the oxidation of NADH ($\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$) by means of pyruvate kinase and lactate dehydrogenase as coupling enzymes. The reaction mixture consisted

* To whom correspondence should be addressed.

of 0.1 M-triethanolamine buffer, pH 7.5, containing 0.05 mM-glucose, 0–4.0 mM-glucose 6-phosphate, 0.01–1.0 mM-ATP, 0.26 mM-NADH, 1.0 mM-phosphoenolpyruvate, 10 mM-MgCl₂, 5 units of lactate dehydrogenase/ml, 5 units of pyruvate kinase/ml and 0.125 µg of glucokinase/ml.

Chemical equilibrium

For determination of the equilibrium concentrations of substrates and products, 5 ml of the reaction mixture containing 1.0 mM-glucose, 0.5–10 mM-ATP, 10 mM-MgCl₂, 4.92 µg of glucokinase/ml and 0.1 M-triethanolamine buffer, pH 7.5, was incubated at 310.2 K for 5 h. Then a 600 µl sample was withdrawn and mixed with 400 µl of 0.02 M-HCl to stop the reaction. Added to the resulting solution were 80 µl of 0.1 M-NaOH, 100 µl of 30 mM-NADP⁺, 48 µl of 0.5 M-MgCl₂ and 1622 µl of 0.1 M-triethanolamine buffer, pH 7.5, the final volume being 2800 µl. The solution was transferred to an absorption cell and the absorbance was measured at 340 nm. Then 100 µl of glucose-6-phosphate dehydrogenase solution (30 units/ml) was added to the solution to let the reaction be completed, and the absorbance was measured again. From the difference in the absorbances, the increase in the NADPH concentration, and thus the concentration of the glucose 6-phosphate formed, was determined.

Rate of the backward reaction

The rates of the backward reaction were measured at 310.2 K by tracing the change in the concentration of glucose, which is one of the products of the reaction. The experiment was carried out in a solution containing 5 mM-glucose 6-phosphate, 5 mM-ADP, 10 mM-MgCl₂, 8.65 µg of glucokinase/ml and 0.1 M-triethanolamine buffer, pH 7.5. At 30 min intervals for 3 h 100 µl portions of the reaction mixture were each taken into 100 µl of 0.12 M-H₃PO₄ to inactivate the enzyme and then this was neutralized with 100 µl of 0.2 M-KOH. The amount of glucose in the solution was analysed by the method of Bergmeyer & Bernt (1981). A 120 µl portion of the above solution was added to 3000 µl of a reaction mixture containing 50 µg of *o*-dianisidine hydrochloride/ml, 1.5 units of peroxidase/ml, 9 units of glucose oxidase/ml and 0.12 M-potassium phosphate buffer, pH 7.5. Then 30 min later the absorbance was measured at 450 nm ($\epsilon_{450} = 386 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Stability of glucokinase

A solution containing 3.75 µg of glucokinase/ml in a 0.1 M-triethanolamine buffer, pH 7.5, was preincubated at 310.2 K. After various incubation periods 100 µl portions of the enzyme solution were taken from the same solution and each was added to 2900 µl of a reaction mixture containing 40 mM-glucose, 1.0 mM-ATP, 1.0 mM-NADP⁺, 10 mM-MgCl₂, 1.0 unit of glucose-6-phosphate dehydrogenase/ml and 0.1 M-triethanolamine buffer, pH 7.5. The rate of formation of glucose 6-phosphate was measured at 310.2 K by the method described above.

Analysis of results

The reaction mechanism was investigated by examining the inhibitory effects of the substrates, glucose and ATP, and the products, glucose 6-phosphate and ADP, on the reaction. For this purpose, the experimental

results obtained for the initial rate were plotted as $[C]/v$ versus $[C]$, where $[C]$ expresses the concentration of the substrate or the product in µM.

Concentrations of complex ions

In reaction mixtures containing Mg²⁺, the adenosine phosphates ATP and ADP are present mainly as forms of MgATP²⁻ and MgADP⁻ respectively. Since these complexes are ligands of the enzyme, the effect of the adenosine phosphates on the reaction should be studied on the basis of the concentrations of the complex ions.

The concentrations of MgATP²⁻, MgADP⁻, protonated complexes and other ions were calculated from the total concentrations of MgCl₂, ATP, ADP and other substances as described by Storer & Cornish-Bowden (1976a), by using the thermodynamic data of Phillips *et al.* (1966) and the data cited in a paper by Langer *et al.* (1977). Calculation showed that the MgATP²⁻ concentration was a high and nearly constant proportion (74 ± 2 %) of the total ATP concentration, and that the MgADP⁻ concentration was also a nearly constant proportion (94 ± 1 %) of the total ADP concentration.

RESULTS

Stability of glucokinase

Fig. 1 shows the remaining activity (the relative rate of reaction) plotted against the incubation period of glucokinase on a semi-logarithmic scale. As shown in the Figure, the experimental results are well correlated with the straight line, in which the slope gives the rate constant of inactivation of the enzyme, $7.04 \times 10^{-4} \text{ h}^{-1}$, this being equivalent to a half-life time of 41.0 days for the enzyme. This result assures us that the effect of the inactivation of the enzyme on the phosphorylation

Fig. 1. Stability of glucokinase

The relative rate of reaction was plotted against the incubation period. A solution containing 3.75 µg of glucokinase/ml in 0.1 M-triethanolamine buffer, pH 7.5, was preincubated at 310.2 K. After various incubation periods 100 µl portions of the enzyme solution were taken and each was added to 2900 µl of a reaction mixture containing 40 mM-glucose, 1.0 mM-ATP, 1.0 mM-NADP⁺, 10 mM-MgCl₂, 1.0 unit of glucose-6-phosphate dehydrogenase/ml and 0.1 M-triethanolamine buffer, pH 7.5. The rate of formation of glucose 6-phosphate was measured at 310.2 K.

reaction was completely negligible in all the present experiments because the measurements of the time course of the change in the glucose 6-phosphate or the MgADP^- concentration were always completed within several minutes.

Dependence of the rate on the MgATP^{2-} concentration

The glucokinase-catalysed rate of glucose phosphorylation, v , was measured at several fixed glucose concentrations, with the MgATP^{2-} concentration varied in the range 3.62–368 μM . In Fig. 2 are shown the experimental results in which the values of $[\text{MgATP}^{2-}]/v$ are plotted against $[\text{MgATP}^{2-}]$. The Figure shows that each series of the experimental results obtained with constant glucose concentrations is well correlated with the straight line, but both the slopes and the intercepts at the ordinate are different from the others. This result suggests that glucose is non-competitive with respect to MgATP^{2-} and that the reaction does not occur by the Ping Pong mechanism.

Dependence of the rate on the MgADP^- concentration

Dependence of the rate on the concentration of the product MgADP^- was examined. Fig. 3 shows the experimental reaction rates measured as a function of MgATP^{2-} concentration at several fixed MgADP^- concentrations, while the glucose concentration was maintained at a constant value of 50 μM , a non-saturating concentration. In Fig. 4 are shown the results obtained at the fixed glucose concentration of 10 mM, the saturating concentration. Though the Figures are not shown in this paper, similar results were obtained at glucose concentrations of 100, 200, 500 and 1000 μM . Both the Figures above and the ones not shown suggest that MgADP^- is a non-competitive inhibitor with respect to MgATP^{2-} irrespective of the glucose concentration.

Fig. 5 shows the experimental results as a plot of $[\text{glucose}]/v$ versus $[\text{glucose}]$. The values of v were measured at several fixed MgADP^- concentrations,

Fig. 2. Dependence of the rate on the MgATP^{2-} concentration

Initial rates, v , were measured at 310.2 K in 0.1 M-triethanolamine buffer, pH 7.5, containing 0.005–1.0 mM-ATP, 10 mM- MgCl_2 , 1.0 mM- NADP^+ , 1 unit of glucose-6-phosphate dehydrogenase/ml and 0.125 μg of glucokinase/ml. Concentrations of glucose were 5 mM (○), 1 mM (Δ), 500 μM (□), 200 μM (●), 100 μM (Δ) and 50 μM (■).

while the MgATP^{2-} concentration was maintained at a constant value of 562 μM , the saturation concentration. The Figure suggests that MgADP^- is an uncompetitive inhibitor with respect to glucose when MgATP^{2-} is maintained at the saturation concentration.

Dependence of the rate on the glucose 6-phosphate concentration

Dependence of the rate on the concentration of another product, glucose 6-phosphate, was also studied. The initial rate was measured at several fixed concentrations of glucose 6-phosphate, while the glucose concentration was maintained at a constant value of 50 μM . Fig. 6, in which the experimental results are shown as a plot of $[\text{MgATP}^{2-}]/v$ versus $[\text{MgATP}^{2-}]$, suggests that the rate is

Fig. 3. Dependence of the rate on the MgADP^- concentration

Initial rates were measured at the low glucose concentration of 50 μM , a non-saturating concentration. Conditions were as for Fig. 2, apart from the following: MgADP^- concentrations were 703 μM (○), 470 μM (Δ), 235 μM (□) and 94.1 μM (●).

Fig. 4. Dependence of the rate on the MgADP^- concentration

Initial rates were measured at the high glucose concentration of 10 mM, the saturating concentration. Conditions were as for Fig. 2, apart from the following: MgADP^- concentrations were 936 μM (○), 470 μM (Δ), 282 μM (□) and 94.1 μM (●).

Fig. 5. Dependence of the rate on the MgADP^- concentration

Initial rates were measured at a constant MgATP^{2-} concentration of $562 \mu\text{M}$, the saturating concentration. Conditions were as for Fig. 2, apart from the following: MgADP^- concentrations were $701 \mu\text{M}$ (●), $468 \mu\text{M}$ (□), $234 \mu\text{M}$ (△) and $93.9 \mu\text{M}$ (○).

Fig. 6. Dependence of the rate on the glucose 6-phosphate concentration

Initial rates were measured in 0.1 M -triethanolamine buffer, pH 7.5, containing $50 \mu\text{M}$ -glucose, 0.01 – 1.0 mM -ATP, 10 mM - MgCl_2 , 0.26 mM -NADH, 1.0 mM -phosphoenolpyruvate, 5 units of lactate dehydrogenase/ml, 5 units of pyruvate kinase/ml and $0.125 \mu\text{g}$ of glucokinase/ml. Glucose 6-phosphate concentrations were 4.0 mM (●), 2.0 mM (□), 0.5 mM (△) and 0 mM (○).

independent of the glucose 6-phosphate concentration and that glucose 6-phosphate is not an inhibitor with respect to either glucose or MgATP^{2-} . This also suggests that the reaction is irreversible.

To confirm this, the equilibrium composition of the reaction mixtures after completion of reaction was analysed. Table 1 shows the glucose 6-phosphate concentrations determined, indicating that the amounts of glucose 6-phosphate produced are equal to those of the total ATP or glucose, both of which were present in the reaction mixtures before the reaction occurred, and that the reaction is essentially irreversible. Furthermore,

Table 1. Relation between the initial total concentration of ATP in reaction mixtures and the equilibrium concentration of glucose 6-phosphate formed

Reaction mixtures (5 ml) containing 1.0 mM -glucose, 0.5 – 10 mM -ATP, 10 mM - MgCl_2 , $4.92 \mu\text{g}$ of glucokinase/ml and 0.1 M -triethanolamine buffer, pH 7.5, were incubated at 310.2 K for 3 h . The glucose 6-phosphate concentration was determined by the method described in the Materials and methods section.

Initial total concn. of ATP (mM)	Equilibrium concn. of glucose 6-phosphate (mM)
0.5	0.521
2.0	1.03
5.0	0.988
10.0	1.00

Table 2. Determined Dalziel coefficients ϕ_i

Determination was based on an M_r of 68000 for glucokinase.

ϕ_0 :	$1.62 \times 10^{-3} \text{ s}$
ϕ_1 :	$8.73 \times 10^{-8} \text{ M} \cdot \text{s}$
ϕ_2 :	$5.21 \times 10^{-8} \text{ M} \cdot \text{s}$
ϕ_3 :	$2.21 \times 10^{-11} \text{ M}^2 \cdot \text{s}$
ϕ_4 :	$3.78 \times 10^{-8} \text{ M} \cdot \text{s}$
ϕ_5 :	$5.84 \times 10^{-4} \text{ s}$
ϕ_6 :	$1.14 \text{ M}^{-1} \cdot \text{s}$

the rate of the backward reaction was measured by using a solution containing 5 mM -glucose 6-phosphate, 5 mM -ADP, and $8.65 \mu\text{g}$ of glucokinase/ml, the enzyme concentration being 69 times that used for obtaining the initial-rate data. No glucose was detected even 3 h after the enzyme was injected into the reaction mixture to start the reaction. This indicated that the backward reaction did not occur at all.

From these results, the phosphorylation of glucose was confirmed to be irreversible.

Mechanism and the kinetic parameters

The results mentioned above suggest that the reaction mechanism of the glucose phosphorylation catalysed by glucokinase from *B. stearothermophilus* is ordered Bi Bi, in which glucose adds to the enzyme before MgATP^{2-} and glucose 6-phosphate is released from the enzyme after the dissociation of MgADP^- , and that the final step in which glucose 6-phosphate is released is irreversible. This mechanism may be given by Scheme 1 in Table 3. Therefore the reaction rate can be expressed by:

$$[E]_0/v = \phi_0 + \phi_1/[\text{glucose}] + \phi_2/[\text{MgATP}^{2-}] + \phi_3/[\text{glucose}][\text{MgATP}^{2-}] + \phi_4[\text{MgADP}^-]/[\text{glucose}][\text{MgATP}^{2-}] + \phi_5[\text{MgADP}^-]/[\text{MgATP}^{2-}] + \phi_6[\text{MgADP}^-] \quad (1)$$

with the Dalziel coefficients ϕ_i .

Table 3. Reaction schemes and their rate equations

A, B, P and Q stand for glucose, MgADP²⁻, MgADP³⁻ and glucose 6-phosphate respectively. Error means the average error obtained by comparing the experimental rate data with the predictions based on the determined rate equation.

Scheme	Rate equation	Remarks
$\begin{array}{l} 1 \quad E \rightleftharpoons EA \rightleftharpoons EAB \rightleftharpoons EPQ \rightleftharpoons EQ \rightarrow E \\ 2 \quad E \rightleftharpoons EA \rightarrow EAB \rightleftharpoons EPQ \rightleftharpoons EQ \rightarrow E \end{array}$	$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_3}{[A][B]} + \frac{\phi_4[P]}{[A][B]} + \frac{\phi_5[P]}{[B]} + \phi_6[P]$	$\left\{ \begin{array}{l} \phi_5 = \phi_2\phi_4/\phi_3 \\ \text{Error } 4.6\% \\ \phi_6 - \phi_5 \text{ are independent} \\ \text{Error } 4.6\% \end{array} \right.$
$3 \quad E \rightleftharpoons EA \rightleftharpoons EAB \rightleftharpoons EPQ \rightleftharpoons EQ \rightarrow E$	$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_3}{[A][B]} + \frac{\phi_4[P]}{[A][B]} + \frac{\phi_5[P]}{[B]} + \phi_6[P] + \frac{\phi_7[P]^2}{[A][B]} + \frac{\phi_8[P]^2}{[B]}$	$\phi_6 - \phi_8 \text{ are independent}$ <p>Error 4.3%</p>
$4 \quad E \rightleftharpoons EA \rightleftharpoons EAB \rightleftharpoons EPQ \rightarrow EP \rightleftharpoons E$	$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_3}{[A][B]} + \frac{\phi_4[P]}{[A][B]} + \frac{\phi_5[P]}{[B]} + \frac{\phi_6[P]}{[A]} + \frac{\phi_7[P]^2}{[A][B]}$	$\phi_6 - \phi_8 \text{ are independent}$ <p>Error 7.9%; ($\phi_4)_{obs} < 0$</p>
$5 \quad E \rightleftharpoons EA \rightleftharpoons EAB \rightleftharpoons EPQ \rightleftharpoons EQ \rightarrow E$	$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_3}{[A][B]} + \frac{\phi_4[P]}{[A][B]} + \frac{\phi_5[P]}{[B]} + \phi_6[P] + \frac{\phi_7[P]}{[A]} + \frac{\phi_8[P]^2}{[A][B]} + \frac{\phi_9[P]^2}{[B]}$	$\phi_6 - \phi_9 \text{ are independent}$ <p>Error 4.3%</p>
$6 \quad E \rightleftharpoons EA \rightarrow EAB \rightleftharpoons EPQ \rightarrow EQ \rightarrow E$	$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_3}{[A][B]} + \frac{\phi_4[P]}{[A][B]} + \frac{\phi_5[P]}{[B]} + \frac{\phi_6[P]}{[A]}$	$\phi_6 - \phi_8 \text{ are independent}$ <p>Error 8.0%</p>
$7 \quad E \rightleftharpoons EA \rightarrow EAB \rightleftharpoons EPQ \rightleftharpoons EQ \rightarrow E$	$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_3}{[A][B]} + \frac{\phi_4[P]}{[A][B]} + \frac{\phi_5[P]}{[B]} + \phi_6[P] + \frac{\phi_7[P]}{[A]}$	$\phi_7 = \phi_1\phi_4/\phi_3$ <p>Error 4.6%</p>

All the experimental measurements of the initial rate (total data points 209), more than half of which are shown in Figs. 2–5, were used to determine the coefficients ϕ_i . These data were fitted to eqn. (1) by the non-linear-regression procedure (the Pawell method) on an ACOS 850 computer. The coefficients thus determined are listed in Table 2.

When the reaction occurs by the ordered Bi Bi mechanism as expressed by Scheme 1 in Table 3, the reaction rate is expressed by eqn. (1) with the Dalziel coefficients. The coefficients are not all independent of each other, but ϕ_5 is related to ϕ_2 , ϕ_3 and ϕ_4 by:

$$\phi_5 = \phi_2\phi_4/\phi_3 \quad (2)$$

The experimental ϕ_5 value determined by comparing directly the experimental reaction rates with eqn. (1) differs by a factor of 6.6-fold from the one calculated by eqn. (2) with the experimental ϕ_2 – ϕ_4 values. We considered that this discrepancy means that the mechanism of the phosphorylation catalysed by the glucokinase from *B. stearothermophilus* does not obey the ordered Bi Bi mechanism expressed by Scheme 1. Therefore we studied the mechanism further.

For about 30 reaction schemes, including the random ternary-complex mechanism (Gregoriou *et al.*, 1981) and the ordered one with inclusion of a non-productive ternary complex, the 'abortive complex' (Storer & Cornish-Bowden, 1977) or the 'dead-end complex', in which glucose and MgADP²⁻ are bound simultaneously to the enzyme, we derived rate equations by making use of the pseudo-steady-state assumption. The rate equations were derived with the aid of a computer program that was developed in our laboratory in BASIC for our PC 9800 series of NEC personal digital computer. Of the rate equations derived, one coincided with eqn. (1), and five were similar to eqn. (1) while they had additional zero to three more terms in the denominator of the rate equations. These rate equations are given in Table 3 with their schemes. Schemes 2–4 include the formation of the 'abortive complex', and Schemes 5–7 include the formation of the 'dead-end complexes'. In the Table, all the Dalziel coefficients ϕ_i were independent of each other

in all the equations, except the one for Scheme 7, in which some of the coefficients are related by:

$$\phi_7 = \phi_1\phi_4/\phi_3 \quad (3)$$

The coefficients for each scheme were determined by fitting the experimental results obtained above to the rate equation by the non-linear-regression procedure. Since in Scheme 4 one of the determined Dalziel coefficients, ϕ_4 , was negative, this scheme should be eliminated. Table 3 also gives the average errors that arose from comparing the predicted rates by using the rate equations given in Table 3 and the Dalziel coefficients ϕ_i determined with the experimental rate data. The Table shows that the rate equation for Scheme 6 had a larger error than those of other schemes. Furthermore in Scheme 7 the determined coefficients ϕ_i do not satisfy the relation expressed by eqn. (3). Each of the remaining three schemes, Schemes 2, 3 and 5, can correlate the experimental rate data with in fact the same error. Therefore it seems difficult to select one and reject the other two for the glucose phosphorylation. However, in general, the more terms the rate equation has, the lower the error of correlation. From this point of view, Scheme 2 seems to be the simplest reasonable model consistent with the observations. The rate equation for this scheme is also expressed by eqn. (1), and the Dalziel coefficients ϕ_i are given in Table 2.

The kinetic parameters can be evaluated from the ϕ_i values determined. The Michaelis constants K_m for glucose and MgATP²⁻ and the maximum velocity V_{max} are compared with those reported for glucokinase and hexokinase in Table 4.

DISCUSSION

Above we have proposed a plausible scheme, which is consistent with the experimental results, for the phosphorylation catalysed by the glucokinase from *B. stearothermophilus*. However, the fact that the experimental Dalziel coefficients ϕ_i did not satisfy the relation given by eqn. (2) may not exclude the ordered Bi Bi mechanism expressed by Scheme 1, if we take into

Table 4. Some kinetic parameters for glucokinase and hexokinase

Source	Temperature (K)	pH	V_{max} (μ mol/s per mg of protein)	K_m (mM)		Reference
				Glucose	MgATP ²⁻	
<i>B. stearothermophilus</i>	310	7.5	9.07	0.054	0.032	Present work
<i>B. stearothermophilus</i>	319	9.0	5.07†	0.52	0.06	Hengartner & Zuber (1973)
<i>Aerobacter aerogenes</i>	298	7.5	64.3†	0.08	0.8	Kamel <i>et al.</i> (1966)
<i>Zymomonas mobilis</i>	298	6.8	6.67	0.8	0.22	Scopes <i>et al.</i> (1985)
<i>Streptococcus mutans</i>	295	7.5	3.30†	0.61	0.21	Porter <i>et al.</i> (1982)
<i>Brevibacterium fuscum</i>	303	7.2	0.92†	1.14	0.787	Saito (1965)
<i>Entamoeba histolytica</i>	298	7.0	—	0.04	0.2	Reeves <i>et al.</i> (1967)
Rat liver	301	7.0	0.117†	16.7	1.0	Parry & Walker (1967)
Rat liver	303	8.0	—	5	0.55	Storer & Cornish-Bowden (1976b)
Yeast*	303	8.5	26.0	0.30	0.475	Noat <i>et al.</i> (1968)
Yeast*	301	7.6	—	0.17	0.2	Fromm & Zewe (1962)

* Hexokinase.

† Specific activity.

consideration the effect of inhibition by Mg^{2+} , ATP^{4-} and/or Mg_2ATP on the reaction. Noat *et al.* (1970) found that ATP^{4-} can be bound on the yeast hexokinase-glucose complex to constitute the 'dead-end' complex. However, the equilibrium concentrations of ATP^{4-} were very low and less than 0.3% of the total ATP concentrations in the present experimental conditions, suggesting a negligible effect of ATP^{4-} inhibition on the reaction. On the other hand, the equilibrium concentrations of Mg^{2+} were considerable and constant at about 6.0 ± 0.7 mM and the amounts of Mg_2ATP were $25 \pm 2\%$ of the total ATP concentration in the present experimental conditions. Noat *et al.* (1970) also showed that both Mg^{2+} and Mg_2ATP possess no affinity for the enzyme and the enzyme-substrate or the enzyme-product complex. Storer & Cornish-Bowden (1977), on the other hand, found a direct inhibitory effect of Mg^{2+} on the reaction catalysed by the rat liver glucokinase. Therefore, to examine whether there is any inhibitory effect of Mg^{2+} and Mg_2ATP on the reaction, some additional experiments were carried out under the conditions shown in Table 5, in which the concentrations of both Mg^{2+} and Mg_2ATP were changed by a factor of 7.15-fold while the concentrations of glucose and MgATP^{2-} were kept constant at 5.0 and 0.748 mM respectively. The reaction rates measured were substantially constant, indicating that there was no inhibitory effect of Mg^{2+} and Mg_2ATP on the rate. Therefore we can conclude that the presence of Mg^{2+} , Mg_2ATP and ATP^{4-} does not cause the discrepancy mentioned above.

The reaction mechanism proposed above is essentially the ordered Bi Bi accompanied by the formation of the non-productive complex glucose-MgADP⁻-enzyme. However, the mechanism of the glucose phosphorylation catalysed by glucokinase depends on the source or the type of the enzyme. Of the glucokinases from other sources, the glucokinase from *S. mutans* (Porter *et al.*, 1982) catalyses the phosphorylation reaction of glucose in accordance with the ordered Bi Bi mechanism. The same mechanism was proposed by Noat *et al.* (1968) for the yeast hexokinase, whereas the random Bi Bi mechanism was also proposed for the yeast hexokinase-catalysed reaction (Fromm & Zewe, 1962; Fromm, 1969). The kinetic behaviour of the glucokinase from rat

liver was also investigated intensively (Parry & Walker, 1967; Storer & Cornish-Bowden, 1976b, 1977; Gregoriou *et al.*, 1981). Parry & Walker (1967) first proposed the random Bi Bi rapid-equilibrium mechanism, in which the interconversion of the central ternary complexes is rate-determining, but found an anomalous behaviour in the double-reciprocal plot of $1/v$ against $1/[\text{glucose}]$ when the glucose concentration was less than 8 mM. Later, Storer & Cornish-Bowden (1976b) revealed that this anomalous behaviour occurred due to a sigmoid or co-operative dependence of the rate on the glucose concentration. Furthermore, Storer & Cornish-Bowden (1976b, 1977) proposed the mnemonical mechanism for the phosphorylation catalysed by the glucokinase from rat liver, in which glucose binds differentially to two forms of the free enzyme that are not in equilibrium under steady-state conditions.

As shown in Table 4, the glucokinase from *B. stearothermophilus* is characterized by low Michaelis constants. The K_m values for glucose for the yeast hexokinase or the glucokinases from all sources except *A. aerogenes* (Kamel *et al.*, 1966) and *E. histolytica* (Reeves *et al.*, 1967) are much higher than that for *B. stearothermophilus*. Furthermore, the K_m values for MgATP^{2-} reported for the glucokinases from all other sources or the yeast hexokinase are much higher than that for *B. stearothermophilus*.

Because of the low K_m values, irreversibility of the reaction and the excellent thermal stability, the glucokinase from *B. stearothermophilus* is suitable for use as an analytical enzyme and an industrial catalyst.

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Table 5. Effect of the concentrations of Mg^{2+} and Mg_2ATP on the reaction rate

Initial rates were measured at 310.2 K in 0.1 M-triethanolamine buffer, pH 7.5, containing 5 mM-glucose, 0.748 mM- MgATP^{2-} , 1 mM-NADP⁺, 1 unit of glucose-6-phosphate dehydrogenase/ml and 0.125 μg of glucokinase/ml. The concentrations of MgCl_2 , Mg^{2+} and Mg_2ATP were changed as listed.

Concn. of MgCl_2 (mM)	Equilibrium concn. of Mg^{2+} (mM)	Equilibrium concn. of Mg_2ATP (mM)	Initial total concn. of ATP (mM)	Reaction rate ($\mu\text{M/s}$)
2.0	0.819	0.0340	0.823	1.10
5.0	2.75	0.114	0.877	1.09
10.0	5.85	0.243	1.00	1.11

REFERENCES

- Bergmeyer, H. U. & Bernt, E. (1981) in *Methods of Enzymatic Analysis*, vol. 3 (Bergmeyer, H. U., ed.), pp. 1205-1215, Verlag Chemie International, Deerfield Beach
- Fromm, H. J. (1969) *Eur. J. Biochem.* **7**, 385-392
- Fromm, H. J. & Zewe, V. (1962) *J. Biol. Chem.* **237**, 3027-3032
- Gregoriou, M., Trayer, I. P. & Cornish-Bowden, A. (1981) *Biochemistry* **20**, 499-506
- Hengartner, H. & Zuber, H. (1973) *FEBS Lett.* **37**, 212-216
- Kamel, M. Y., Allison, D. D. & Anderson, R. L. (1966) *J. Biol. Chem.* **241**, 690-694
- Langer, R. S., Gardner, C. R., Hamilton, B. K. & Colton, C. K. (1977) *AIChE J.* **23**, 1-10
- Noat, G., Ricard, J., Borel, M. & Got, C. (1968) *Eur. J. Biochem.* **5**, 55-70
- Noat, G., Ricard, J., Borel, M. & Got, C. (1970) *Eur. J. Biochem.* **13**, 347-363
- Parry, M. J. & Walker, D. G. (1966) *Biochem. J.* **99**, 266-274
- Parry, M. J. & Walker, D. G. (1967) *Biochem. J.* **105**, 473-482
- Phillips, R. C., George, P. & Rutman, R. J. (1966) *J. Am. Chem. Soc.* **88**, 2631-2640
- Porter, E. V., Chassy, B. M. & Holmlund, C. E. (1982) *Biochim. Biophys. Acta* **709**, 178-186
- Reeves, R. E., Montalvo, F. & Sillero, A. (1967) *Biochemistry* **6**, 1752-1760
- Saito, N. (1965) *J. Biochem. (Tokyo)* **57**, 363-371

- Scopes, K., Testolin, V., Storer, A., Griffiths-Smith, K. & Algar, E. M. (1985) *Biochem. J.* **228**, 627–634
- Slein, M. W., Cori, G. T. & Cori, C. F. (1950) *J. Biol. Chem.* **186**, 763–780
- Storer, A. C. & Cornish-Bowden, A. (1974) *Biochem. J.* **141**, 205–209
- Storer, A. C. & Cornish-Bowden, A. (1976*a*) *Biochem. J.* **159**, 1–5
- Storer, A. C. & Cornish-Bowden, A. (1976*b*) *Biochem. J.* **159**, 7–14
- Storer, A. C. & Cornish-Bowden, A. (1977) *Biochem. J.* **165**, 61–69
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