The Kinetics and Mechanism of a Reaction Catalyzed by Bacillus steareothermophilus Phosphoglucone Isomerase

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The initial rates of isomerization between glucose 6-phosphate and fructose 6-phosphate catalyzed by Bacillus steareothermophilus phosphoglucone isomerase (PGI) were measured in both the forward and reverse reactions. Although B. steareothermophilus PGI is a tetrameric enzyme, the reaction rate vs substrate concentration curves for both reactions exhibited Michaelis-Menten kinetic behavior. This was confirmed by the Hill plot which gave the Hill coefficient of 1.0 for both reactions. Based on the above experimental results and another experimental result that the number of substrate or product binding sites on the PGI molecule was 4, we propose a reaction scheme which is able to explain Michaelis-Menten kinetic behavior of this oligomeric enzyme, and determine the kinetic parameters.

[Key words: kinetics, mechanism, tetrameric enzyme, phosphoglucone isomerase, glucose 6-phosphate, fructose 6-phosphate]

Phosphoglucone isomerase (PGI: D-glucose 6-phosphate ketoisomerase; EC 5.3.1.9) is widely distributed in nature and catalyzes the isomerization reaction between glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P). PGI is physiologically very important as it is an enzyme of the glycolytic pathway. Furthermore, PGI plays an important role in the industrial production of fructose 1,6-diphosphate (FDP), which is expected to be useful for various medical applications, from glucose (1).

Purification of PGI has been achieved from mesophilic sources, such as human erythrocyte (2, 3), spinach leaves (4), brewer's yeast (5, 6), and rabbit muscle (7), as well as from thermophilic sources, such as Bacillus caldotenax (8) and Bacillus stearethermophilus (9). For analytical or industrial applications thermophilic PGI is suitable due to its high thermostability. There is some information available concerning the kinetics and mechanism of the reaction catalyzed by the mesophilic PGI, however almost none exists concerning the kinetics and mechanism of the reaction catalyzed by the thermophilic PGI.

In the present work, the initial rates of both the forward and reverse reactions of the isomerization between G6P and F6P catalyzed by PGI of the thermophilic organism B. stearethermophilus at pH 8.0 and 30°C under various G6P or F6P concentration conditions, and the time courses of the increase in the G6P concentration produced by the reverse reaction were measured. Furthermore, experiments concerning the molecular mass of the enzyme and the number of substrate or product binding sites per molecule were performed. Based on these experimental results, we propose a reaction mechanism.

MATERIALS AND METHODS

Materials  B. stearethermophilus PGI was obtained from Unitika Ltd. (Osaka). The protein marker kit for SDS-polyacrylamide gel electrophoresis was purchased from New England Biolabs, Inc. (Massachusetts, USA), and that for gel chromatography was from Serva Feinbiochemica GmbH. & Co. (Heidelberg, Germany). Monosodium salt of G6P and disodium salt of F6P were purchased from Sigma (St. Louis, MO, USA), D-[-1-14C]-glucose 6-phosphate from NEN (Tokyo), and glucose 6-phosphate dehydrogenase (G6PDH), NADP+, triethanolamine (TEA), MgCl2, and other chemicals from Wako Pure Chemical Ind. Ltd. (Osaka).

Solutions were made up in 0.1 M TEA buffer solutions containing 0.1 M HCl and adjusted to pH 8.0 using an aqueous KOH solution.

Stability of B. stearethermophilus PGI  To study the stability of B. stearethermophilus PGI, a solution containing 2.1 x 10^{-5} mM PGI in 0.1 M TEA buffer (pH 8.0) was incubated at 30°C. After various incubation periods, 50 μl aliquots of the enzyme solution were taken and each was added to 2950 μl of a reaction mixture containing 8.40 mM F6P, 1.0 mM NADP+, 2.0 U/ml G6PDH in 0.1 M TEA buffer (pH 8.0). The activity of the enzyme was measured by determining the rate of G6P formation by the reverse reaction at 30°C using the method described below.

Gel chromatography and SDS-polyacrylamide gel electrophoresis  Gel chromatography experiments were performed using a 1100/160 Superdex 200 p.g. gel filtration column which was equilibrated with 10 mM Tris-HCl buffer containing 150 mM NaCl. Two ml of a 1.32 x 10^{-4} mM PGI solution was loaded onto the column and was eluted at a rate of 1 ml/min at 4°C with the same buffer. As a standard, a mixture containing the marker proteins DNP-l-alanine (molecular mass: 0.225 kDa), cytochrome C (12.3 kDa), myoglobin (17.8 kDa), chymotrypsinogen A (25 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), rabbit muscle aldolase (160 kDa), bovine catalase (240 kDa), and horse spleen ferritin (450 kDa) was applied to the same column.

SDS-polyacrylamide gel electrophoresis was performed

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at 10 mA for 4 h according to the method of Weber and Osborn (10) at a 7% gel concentration. As a standard, a mixture containing the marker proteins MBP, β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa) and triose-phosphate isomerase (32.5 kDa), was used.

**The number of substrate or product binding sites on B. stearothermophilus PGI** The number of substrate or product binding sites on PGI was measured by the dialysis equilibrium method (11). The experiments were carried out in a dialysis equilibrium apparatus consisting of two jacketed acrylic resin cells each with a working volume of 0.7 ml. Temperature-regulated water was circulated inside the jackets to maintain the temperature at 30°C. A Teflon-coated magnetic stirrer bar was placed in each of the cells to stir the liquid inside the cells. One of the compartments (compartment I) was filled with 0.1 M TEA buffer (pH 8.0) containing 52 µM PGI, while the other (compartment II) was filled with 0.1 M TEA buffer containing 0.15-10 mM G6P which contained [1-14C]-G6P. The ratio of the labeled G6P molar concentration to the total G6P molar concentration ranged from 0.00021 to 0.19 when the buffers containing labeled G6P were prepared. As in previous investigations, Visking Cellophane dialysis tubing was used as a membrane for separating the two compartments. Twenty-five µl of liquid sample was taken from each compartment for determination of the concentration of labeled G6P using a liquid scintillation counter (Wallac 1409 model, Pharma- cia). In the present experiment, the total concentrations of D-[1-14C]-G6P and D-[1-14C]-F6P, and of G6P and F6P were measured because F6P was produced by the catalytic action of the enzyme. When equilibrium was attained between the solutions in the two compartments, the next run was carried out by replacing the solution in compartment II with fresh ligand (G6P) solution of a different concentration. A small amount of the enzyme solution was added to compartment I to compensate for the sample withdrawn for analysis. The time required to attain the equilibrium state was 4 d. The total concentration of ligands (G6P and F6P) bound to the enzyme was determined by the difference in concentrations between the two compartments.

**The initial rates of the forward reaction** To obtain the initial rates of the forward reaction of the isomerization between G6P and F6P (the reaction in which G6P is the substrate and F6P is the product), the F6P measurements were by the colorimetric method described by Roe (12). A 10 ml-volume vial, with a jacket in which temperature-regulated water at 30°C was circulated, was used as the reactor. Five ml of a substrate solution containing 0.3-25 mM G6P in 0.1 M TEA buffer (pH 8.0) was stirred with a Teflon-coated magnetic stirrer bar. After the solution temperature reached 30°C, 100 µl of the enzyme solution (the final concentration was 1.97 x 10^{-7} mM PGI) was added to the solution to initiate the reaction. At appropriate time intervals, 200 µl aliquots of liquid samples were taken and each was mixed promptly with 0.8 ml of H3PO4 (pH 1.6) to stop the reaction. Then, to these solutions NADP+ and G6PDH were added (the final concentrations were 1 mM and 0.1 U/ml, respectively), and the concentrations of G6P produced were measured by following the absorbance change due to NADPH formation at 340 nm using the spectrophotometer.

**RESULTS AND DISCUSSION**

**Stability of B. stearothermophilus PGI** Figure 1 shows the experimental results as a plot of the relative remaining activity vs the incubation period on a semi-logarithmic scale. As shown in the figure, the experimental results were correlated well with a straight line generated by the linear regression method. The slope of the straight line gave the inactivation rate constant of the enzyme, k = -1.76 x 10^{-4} h^{-1}, which was equivalent to a half-life of 164 d. This result assured us that the effect of enzyme inactivation on the isomerization reaction was negligible in all the reaction experiments because the measurements of the time courses of the G6P or F6P concentration were always completed within 90 min. However, 3.7% inactivation of PGI may have occurred during the dialysis equilibrium experiments where the longest experimental period was 12 d. We do not believe that this inactivation led to significant errors in the determination of the number of binding sites of the enzyme.

**Gel chromatography and SDS-polyacrylamide gel electrophoresis** The experimental elution volumes of DNP-l-alanine, cytochrome C, myoglobin, chymotrypsinogen A, egg albumin, bovine serum albumin, rabbit muscle aldolase, bovine catalase, and horse spleen ferritin were 126.2, 98.0, 94.4, 91.8, 82.0, 75.4, 67.8, 65.8, and 44.0 ml, respectively. These experimental results are well described by the following equation:

\[ \ln m = 17.7078 - 0.0842 V \]
in 0.1 M-TEA buffer (pH 8.0) was incubated at 30°C. After various incubation periods, 50 µl aliquots of the enzyme solution were taken and each was added to 2950 µl of a reaction mixture containing 8.4 mM F6P, 1.0 mM NADP+, 2.0 U/ml G6PDH in 0.1 M TEA buffer (pH 8.0). The activity of the enzyme was measured by determining the rate of G6P formation by the reverse reaction at 30°C using the method described in the experimental section.

The number of substrate or product binding sites of PGI. In order to clarify the mechanism of the isomerization reaction between G6P and F6P, it is very important to know the number of substrate or product binding sites of B. stearothermophilus PGI. The number of binding sites on a protein for ligands (small molecules or ions) can be determined using the following equation (13, 14).

\[
[F](n- [B])/[B] = K_d
\]  
(3)

Here, \( K_d \) represents the dissociation constant, [F] the concentration of free ligand, [B] the concentration of bound ligand per total protein concentration and \( n \) the number of binding sites. By plotting the experimental results as [B]/[F] vs [B], the number of binding sites and the dissociation constant can be obtained from the intercept of the abscissa and the slope of the straight line, respectively.

This method is valid in principle when small molecules or ions bind to proteins without any catalytic action by the proteins after binding. However, several investigators (11, 15, 16) successfully used this method to determine the number of substrate or product binding sites of an enzyme.

There are three possibilities concerning the number of the binding sites of the tetrameric enzyme PGI. First, there are four binding sites per molecule. In this case, the enzyme works as a tetrameric enzyme as it consists of four active units. Second, there are two binding sites, and the enzyme works as a dimer-like enzyme. Third, there is only one binding site so that the tetrameric PGI works as a monomer-like enzyme.

The results of the dialysis equilibrium experiments are given in Table 1. The values of \([B]\) and \([F]\) calculated from the data are shown in Fig. 3 as a plot of \([B]/[F] vs [B]. The largest experimental \([B] value was 3.1. It is clear that the number of the binding sites on the enzyme is neither one nor two. The figure shows that the data points may be correlated by a straight line or a convex downward curve. The straight line which correlates the data points intersects the abscissa at a \([B] value of about 3.4. However, a convex downward curve will intersect at a \([B] value which is greater than 3.4. Because there is no possibility that the number of the binding sites is 3, it can be concluded that the number of the binding site on B. stearothermophilus PGI is four, that is, one binding site per subunit.
TABLE 1. Results of dialysis equilibrium experiments

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Total amount of ligands$^b$ in compartments I and II (μmol)</th>
<th>Total count in compartment I (dpm)$^a$</th>
<th>Total count in compartment II (dpm)$^a$</th>
<th>Total concentration of ligands$^b$ in compartment I (M)</th>
<th>Total concentration of ligands$^b$ in compartment II (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.93</td>
<td>8.00 × 10$^5$</td>
<td>7.50 × 10$^5$</td>
<td>5.03 × 10$^{-3}$</td>
<td>4.87 × 10$^{-3}$</td>
</tr>
<tr>
<td>2</td>
<td>1.37</td>
<td>6.98 × 10$^5$</td>
<td>6.22 × 10$^5$</td>
<td>1.03 × 10$^{-3}$</td>
<td>9.22 × 10$^{-4}$</td>
</tr>
<tr>
<td>3</td>
<td>0.646</td>
<td>3.32 × 10$^5$</td>
<td>2.85 × 10$^5$</td>
<td>4.97 × 10$^{-4}$</td>
<td>4.26 × 10$^{-4}$</td>
</tr>
<tr>
<td>4</td>
<td>0.52$^/$</td>
<td>5.99 × 10$^5$</td>
<td>4.85 × 10$^5$</td>
<td>2.82 × 10$^{-4}$</td>
<td>2.82 × 10$^{-4}$</td>
</tr>
<tr>
<td>5</td>
<td>0.180</td>
<td>2.99 × 10$^5$</td>
<td>2.37 × 10$^5$</td>
<td>1.44 × 10$^{-4}$</td>
<td>1.14 × 10$^{-4}$</td>
</tr>
<tr>
<td>6</td>
<td>0.108</td>
<td>1.30 × 10$^5$</td>
<td>9.91 × 10$^4$</td>
<td>8.72 × 10$^{-5}$</td>
<td>6.68 × 10$^{-5}$</td>
</tr>
</tbody>
</table>

$^a$ dpm = disintegrations per minute.

$^b$ ligands = both the substrate G6P and the product F6P.

Columns 3 and 4 show the total counts [dpm] for the labeled ligands in compartments I and II, respectively. Column 5 shows the total concentration of the free and bound ligands in compartment I. Column 6 shows the total concentration of the free ligands in compartment II. From the experimental results of Run no. 1, the values of [B] and [F] can be calculated as follows: as the working volume of each compartment was 0.7 ml, the total concentration of the ligands in compartment I is calculated as 

\[(8.00 \times 10^4)/(8.00 \times 10^4 + 7.75 \times 10^4) \times 6.93)/0.7/1000 = 5.03 \times 10^{-3} \text{ M}\]

The total concentration of the ligands in compartment II, which is equal to [F], is calculated in the same way. The total concentration of ligands bound to the enzyme is given by the difference between the concentrations shown in columns 5 and 6. Using an enzyme concentration of 52 nM, [B] is calculated as:

\[[B] = (5.03 \times 10^{-3} - 4.87 \times 10^{-3})/52 \times 10^{-6} - 3.1\]

For other runs, the values of [B] and [F] can be calculated in the same way.

**Initial reaction rates of the forward and reverse reactions**

Figure 4 shows the experimental forward reaction rates as a plot of the reaction rate $v$ vs the G6P concentration [G6P], and Fig. 5 the experimental reverse reaction rates as a plot of $-v$ vs the F6P concentration [F6P]. The lines appearing in the figure are the theoretical curves and will be explained later. Both the figures show that the $v-\left[S\right]$ curves are hyperbolic, indicating that both the reactions exhibit Michaelis-Menten kinetic behavior.

To check Michaelis-Menten kinetic behavior, the data shown in Figs. 4 and 5 were replotted as $v/\left(v_{\text{max}} - v\right)$ vs [F6P] or [G6P] on a logarithmic scale in Figs. 6 and 7, respectively. In both figures, the data could be correlated well with a straight line. From the slopes of the straight lines, the Hill coefficients were determined to be 1.0 in both the cases. This demonstrates that the $v-\left[S\right]$ curves of the forward and reverse reactions exhibit Michaelis-Menten kinetic behavior despite the fact that *B. stearothermophilus* PGI is a tetrameric enzyme consisting of four subunits, each of which has one binding site for substrate or product. Furthermore, the $v-\left[S\right]$ curves of the forward and reverse reactions of isomerization between G6P and F6P catalyzed by *B. stearothermophilus* PGI exhibited Michaelis-Menten kinetic behavior. This was confirmed by the Hill plots which give the Hill coefficient of 1.0 for both the reactions.

The $v-\left[S\right]$ curves of the reactions catalyzed by oligomeric enzymes usually exhibit sigmoidal behavior and sometimes exhibit Michaelis-Menten kinetic behavior. The sigmoidal behavior of the $v-\left[S\right]$ curves of the reactions catalyzed by oligomeric enzymes was fully analyzed by Monod et al. (17) and Koshland et al. (18). However, reverse reaction. As can be seen in the figure, the higher the initial concentration of F6P, the longer it takes to reach equilibrium. However, 90 min was a sufficient period of time for the four initial F6P concentrations tested to reach equilibrium.

The lines appearing in the figure are the theoretical values which will be explained later.

**Mechanism**

As shown above, *B. stearothermophilus* PGI is a tetrameric enzyme consisting of four subunits, each of which has one binding site for substrate or product. Furthermore, the $v-\left[S\right]$ curves of the reactions catalyzed by oligomeric enzymes was fully analyzed by Monod et al. (17) and Koshland et al. (18). However,
FIG. 5. Initial rates of the reverse reaction catalyzed by B. stearothermophilus PGI. The initial rates of the reverse reaction were measured at 30°C in a total volume of 3.0 ml of 0.1 M TEA buffer (pH 8.0) containing 0.05-7.0 mM F6P, 1.0 mM NADP+, 2.0 U/ml G6PDH, and 5.26 × 10⁻⁶ mM PGI. The formation of G6P was followed indirectly by measuring the absorbance change due to NADPH formation at 340 nm (ε₆₂₂=6220 M⁻¹ cm⁻¹) using a Shimadzu UV-2100 spectrophotometer. The solid line was calculated using Eq. 7 and the kinetic parameters kₕ and Kₚ listed in Table 2.

Michaelis-Menten behavior of the reactions catalyzed by oligomeric enzymes has not yet been analyzed in full. Ishikawa et al. (19) studied the effects of the reaction schemes and the kinetic parameters on the co-operativity of the reaction catalyzed by a dimeric enzyme. For the reaction S=P catalyzed by a dimeric enzyme, the reaction schemes were considered on the basis of the KNF model (18). The rate equation for each of the possible schemes was derived on the basis of the combined steady-state and rapid-equilibrium assumptions. They found that even if interaction occurred between the distinct protomers or subunits, the sigmoidal rate behavior was not necessarily observed. On the other hand, the deviation of the v-[S] curve from the hyperbola associated with Michaelis-Menten kinetic behavior could be observed even if there was no interaction between the distinct protomers or subunits. Thus, they found that Michaelis-Menten kinetic behavior could be observed to occur even for a reaction catalyzed by an oligomeric enzyme.

Based on the above theoretical analysis, Ishikawa et al. (20) clarified the kinetics and mechanism of the ATP regeneration reaction catalyzed by B. stearothermophilus acetate kinase, which consists of four subunits but can be treated as a dimer-like enzyme because its functional unit consists of two subunits (21). The rate equation derived based on the Random Bi Bi mechanism and on the KNF model offered a good explanation of the experimental v-[S] curves in which the forward reaction exhibited Michaelis-Menten behavior while the reverse reaction exhibited sigmoidal behavior. We believe that this was the first investigation which clarified wholly the kinetics and mechanism of a reversible reaction catalyzed by an oligomeric enzyme.

FIG. 6. Hill plot of the initial rates of the forward reaction catalyzed by B. stearothermophilus PGI. The data shown in Fig. 4 were replotted as v/(vₘₐₓ - v) vs [G6P]. The data in the region where 0.1 < v/(vₘₐₓ - v) < 10 were correlated by a straight line using the linear regression method. From the slope of the straight line, the Hill coefficient was determined to be 1.0.

FIG. 7. Hill plot of the initial rates of the reverse reaction catalyzed by B. stearothermophilus PGI. The data shown in Fig. 5 were replotted as v/(vₘₐₓ - v) vs [F6P]. The data in the region where 0.1 < v/(vₘₐₓ - v) < 10 were correlated by a straight line using the linear regression method. From the slope of the straight line, the Hill coefficient was determined to be 1.0.

FIG. 8. Time courses of the increase in G6P concentration. The time courses of the increase in G6P concentration produced by the reverse reaction were measured at 30°C in 0.1 M TEA buffer (pH 8.0) containing 5.26 × 10⁻⁶ mM PGI. F6P concentrations were 2 mM (■), 3 mM (●), 5 mM (○) and 10 mM (▲). At appropriate time intervals, 200 pl aliquots of the liquid samples were taken and each was mixed promptly with 0.8 ml of H₃PO₄ (pH 1.6) to stop the reaction. Then, to these liquid samples NADP+ and G6PDH were added (the final concentrations were 1 mM and 0.1 U/ml, respectively), and the concentrations of G6P produced were measured spectrophotometrically by following the absorbance change due to NADPH formation at 340 nm.
In the present work, efforts were made to find a plausible reaction scheme that could explain Michaelis-Menten kinetic behavior of the isomerization reaction between G6P and F6P catalyzed by *B. stearothermophilus* PGI, a tetrameric enzyme. According to Ishikawa et al. (19), the rate equations for the single substrate-single product/dimeric enzyme system reduced to the Michaelis-Menten rate equation under some special conditions if the reaction scheme did not include the reaction step between the complexes ESS and EPP. Therefore, a similar approach was adopted in this single substrate-single product/tetrameric enzyme system. Figure 9 shows a simplified scheme in which the reactions between the complexes ESS and EPP, ESSS and EPPP, and ESSSS and EPPPPP are neglected. In the scheme, E represents a tetrameric enzyme consisting of four subunits, of which one has one binding site for S or P, and S and P are the substrate (G6P) and the product (F6P), respectively. ES and EP represent complexes in which one of the subunits is bound to S and to P, respectively. ESP represents a complex in which one of the subunits is bound to S and another one is bound to P.

As in the case of the single substrate-single product/dimeric enzyme system (19), further simplifications were introduced in order to reduce the number of kinetic constants to the same as or less than that of the experimentally obtainable kinetic parameters: The binding rates of the substrate to both the free enzyme and the enzyme complex, and the dissociation rates of the product from the enzyme complex are much faster than those of the reactions in which a change in the molecular structure of the substrate or the product occurs. Therefore the rapid-equilibrium assumption was applied to steps 1–20, and the steady state assumption was applied to steps 21–30 in the scheme shown in Fig. 9.

The rate equation for the scheme can be derived by the method of King and Altman (22) or others. However, because of the number of reaction steps in the scheme proposed in the present work, the use of a computer program developed by the present authors, which is an advanced version of the previous program (23), is necessary. The rate equation derived was as follows.

\[
v = \frac{-d[S]}{dt} = \frac{d[P]}{dt}
\]

\[
\]

\[\text{(4)}\]

In Eq. 4, there are 20 equilibrium constants \(K_i\) (\(i = 1\)–\(20\)) for steps 1–20, and 20 rate constants consisting of 10 rate constants \(k_f\) (\(j = 21\)–\(30\)) for the forward reactions and 10 rate constants \(k_r\) (\(j = 21\)–\(30\)) for the reverse reaction of steps 21–30. The rate constants and the equilibrium constants as described by Segel (24). As the enzyme consists of four subunits, there are four sets of the intrinsic constants, that is, one for each of the four subunits. However, if the active sites are identical and completely independent of each other, the existence of a substrate or a product at one site will have no effect on the binding properties of the other sites (24). In this special case, it is sufficient to use only one set of the four intrinsic constants, \(k_r, k_f, K_S\), and \(K_P\). The effective constants \(K_{Sj}\) (\(j = 1–10\)), \(K_{pj}\) (\(j = 1–10\)), \(k_fj\) (\(j = 21–30\)), \(k_rj\) (\(j = 21–30\)) were related to the intrinsic constants \(K_S\), \(K_P\), \(k_r\), and \(k_f\), respectively, by a similar method to that reported by Segel (24), and the relationships obtained were substituted into Eq. 4 to give the following equation.

\[
\]

\[\text{(5)}\]

Equation 5 has only two rate constants \(k_f\) and \(k_r\) and two equilibrium constants \(K_S\) and \(K_P\). However, it is noteworthy that the rate equation derived in the present work is quite different from that for a reversible reaction catalyzed by a monomeric enzyme (25). When \([P]=0\), Eq. 5 reduces to the Michaelis-Menten rate equation as given by:

\[
v = \frac{-d[S]}{dt} = \frac{4K_k[E][S]}{1/K_c + [S]}
\]

\[\text{(6)}\]

Similarly, when \([S]=0\), the rate equation 5 also reduces to the Michaelis-Menten rate equation as follows:

\[
v = \frac{-d[P]}{dt} = \frac{4K_k[E][P]}{1/K_p + [P]}
\]

\[\text{(7)}\]
by Eqs. 6 and 7 using a non-linear regression procedure (the Pawell method).

At equilibrium, Eq. 5 yields the following relationship:

\[
\frac{[P]_e}{[S]_e} = \frac{K_{SP}}{K_{PS}}
\]  

(8)

The intrinsic kinetic parameters \(k_f\), \(k_r\), \(K_S\) and \(K_P\) were determined by fitting the experimental initial reaction rates shown in Figs. 4 and 5 to the rate equations given by Eqs. 6 and 7 using a non-linear regression procedure (the Pawell method). The parameters thus determined are listed in Table 2.

Equation 8, which was derived from the rate equation for the proposed scheme, implies that the value of \([P]_e/[S]_e\) must be constant irrespective of the initial concentrations of P (F6P) or S (G6P). Substitution of the experimental kinetic parameters listed in Table 2 into Eq. 8 gives the \([P]_e/[S]_e\) value of 0.46. Table 3 lists the experimental \([P]_e/[S]_e\) values which were directly calculated from the equilibrium concentrations of F6P and G6P determined from the time courses of \([G6P]_e\) shown in Fig. 8. The \([P]_e/[S]_e\) values were found to be constant at an average value of 0.45 irrespective of the initial F6P concentrations. Since this average value was in close agreement with the above value of 0.46, the reaction scheme proposed in the present work was demonstrated to offer an adequate explanation of Michaelis-Menten kinetic behavior.

The solid line in Fig. 4 shows the theoretical line calculated based on the rate equation Eq. 6, with the kinetic parameters listed in Table 2. The initial rate data in this figure were obtained using solutions containing only F6P as the substrate. Similarly, the solid line in Fig. 5 shows the theoretical line calculated based on Eq. 7 and is compared with the data obtained by using solutions containing only F6P as the substrate. Agreement between the theoretical lines and experimental data is satisfactory in both the figures.

Using the kinetic parameters shown in Table 2, the theoretical values of the time courses of the increase in the G6P concentration formed by the reverse reaction were calculated based on Eq. 5 using the Runge-Kutta method. In Fig. 8, the theoretical values are shown as solid lines and are compared with the experimental time courses of the G6P concentration. All the data were obtained using solutions containing both G6P and F6P, though the solutions initially contained only F6P. The theoretical lines and experimental data are also in good agreement in this figure.

From the above experimental results, we can conclude that despite the fact that \(B. \text{stearothermophilus}\) PGI is a tetrameric enzyme, its kinetic behavior can be explained by the Michaelis-Menten rate equation.

In the present work, we clarified the kinetics and the mechanism of a single substrate-singie product reaction catalyzed by a tetrameric enzyme. To the best of our knowledge, the present study was the second investigation that wholly clarified the kinetics and mechanism of a reversible reaction catalyzed by an oligomeric enzyme. We believe that the present study contributes to the analysis of the kinetics and mechanism of reactions catalyzed by other oligomeric enzymes and the rational design of a reactor for the enzymatic production of FDP from glucose.

**NOMENCLATURE**

[B] : concentration of bound substrates or ligands per total protein concentration, M

E : enzyme

\[E]_0 : \text{initial enzyme concentration, M} \]

ES, ESS, ESSS, ESSSS, EP, EPP, EPPP, EPPP, ESP, ESSP, ESSSP, ESPPP, ESSPP : complexes of enzyme and one or more substrates (S) and/or products (P)

[F] : concentration of free molecules or ligands, M

\(k_f\) : intrinsic rate constant of the forward reaction of the slow steps, s\(^{-1}\)

\(k_r\) : effective rate constant for the forward reaction of slow step \(j (j = 21-30)\), s\(^{-1}\)

\(k_r\) : intrinsic rate constant of the reverse reaction of the slow steps, s\(^{-1}\)

\(k_{ri}\) : effective rate constant for the reverse reaction of slow step \(j (j = 21-30)\), s\(^{-1}\)

\(k_{ij}\) : effective equilibrium constant of rapid step \(j (j = 1-20)\) (for example, \(K_1 = [ES]/[E][S], K_2 = [EP]/[E][P], \ldots\)), M\(^{-1}\)

\(k_d\) : inactivation rate constant of enzyme, h\(^{-1}\)

\(K_d\) : dissociation constant, M

\(K_P\) : intrinsic equilibrium constant of the steps in which the product binds or dissociates, M\(^{-1}\)

\(K_S\) : intrinsic equilibrium constant of the steps in which the substrate binds or dissociates, M\(^{-1}\)

\(m\) : molecular mass, Da

\(n\) : number of substrate or product binding sites of enzyme, —

P : product (F6P)

\([P]\) : concentration of product, M

S : substrate (G6P)

\([S]\) : concentration of substrate, M

\(t\) : reaction time, s

\(v\) : reaction rate, M\(\cdot\)s\(^{-1}\)

\(V_{el}\) : elution volume, ml

\(v_{max}\) : maximum reaction rate, M\(\cdot\)s\(^{-1}\)

**TABLE 2.** Kinetic parameters determined by using the data shown in Figs. 4 and 5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_f)</td>
<td>8.46 \times 10^6 s(^{-1})</td>
</tr>
<tr>
<td>(K_S)</td>
<td>4.90 \times 10^6 M(^{-1})</td>
</tr>
<tr>
<td>(k_r)</td>
<td>1.83 \times 10^6 s(^{-1})</td>
</tr>
<tr>
<td>(K_P)</td>
<td>4.90 \times 10^6 M(^{-1})</td>
</tr>
</tbody>
</table>

The kinetic parameters were determined by fitting the experimental initial reaction rates shown in Figs. 4 and 5 to the rate equations given by Eqs. 6 and 7 using a non-linear regression procedure (the Pawell method).

**TABLE 3.** The experimental equilibrium concentrations of G6P and the equilibrium concentration ratios obtained from the data in Fig. 8

<table>
<thead>
<tr>
<th>[G6P]_0 (mM)</th>
<th>[G6P]_e (mM)</th>
<th>Experimental [F6P]_e/[G6P]_e</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.88</td>
<td>0.454</td>
</tr>
<tr>
<td>5</td>
<td>3.44</td>
<td>0.454</td>
</tr>
<tr>
<td>3</td>
<td>2.06</td>
<td>0.457</td>
</tr>
<tr>
<td>2</td>
<td>1.42</td>
<td>0.415</td>
</tr>
<tr>
<td>av.</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

The experimental equilibrium concentration ratios were directly calculated from the equilibrium concentrations of F6P and G6P determined from the time courses of the increase in G6P concentration shown in Fig. 8.
ACKNOWLEDGMENTS

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