Kinetic Studies of a Recombinant Cellulobiose Phosphorylase (CBP) of the Clostridium thermocellum YM4 Strain Expressed in Escherichia coli

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A cellulobiose phosphorylase (CBP) cloned from the Clostridium thermocellum YM4 strain was purified to homogeneity, and the reaction mechanisms of both the phosphorolytic and synthetic reactions were studied in detail. The enzyme reaction proceeded via an ordered bi bi mechanism, in which Pi bound to the enzyme prior to D-cellobiose and then G 1-P was released after D-glucose. The order of substrate binding was different from that of CBP from Cellvibrio gilvus, which bound to cellobiose prior to Pi. In the synthetic reaction, the enzyme showed three times higher activity with β-D-glucose than with α-D-glucose, and also showed weak activity with 1,5-anhydro-D-glucitol, indicating that the β-anomeric hydroxyl group of D-glucose is highly required. However, even when it is removed enzyme activity remains. The substrate specificity and kinetic studies revealed that the configurations of the C3 and C4 hydroxyl groups were strictly required for the enzyme activity, whereas those of C2 and C6 could be substituted or deleted. The mechanism of substrate inhibition by D-glucose was studied in detail and it was concluded that D-glucose competed with G 1-P for its binding site in the synthetic reaction.

Key words: anomeric hydroxyl group, cellulobiose phosphorylase, Clostridium thermocellum YM4, competitive substrate inhibition, ordered bi bi mechanism.

Cellobiose phosphorylase (CBP; cellulobiose:orthophosphate α-D-glucosyl transferase, EC 2.4.1.20) catalyzes the reversible phosphorolysis of D-cellobiose to a-D-glucose-1-phosphate (G 1-P) and D-glucose. It has been found in several microorganisms such as Cellvibrio gilvus (1), Clostridium stercorarium (2), Cellulomonas (3, 4), Fomes annosus (5), Ruminococcus flavefaciens (6), Thermotoga neapolitana (7), and Clostridium thermocellum strain 643 (8). The enzyme is produced intracellularly in these microorganisms and plays an essential role in the energy-efficient catabolism of cellobiose in the cytoplasm (9). It is known that the enzyme is absolutely specific for the cleavage and synthesis of β(1→4) glucosidic bonds but exhibits a relaxed specificity with respect to the reducing sugar that functions as a glucosyl acceptor in the synthetic reaction (10). Using this relaxed substrate specificity, a number of novel β-1,4-glucosyl products have been synthesized (11–13).

Clostr. thermocellum YM4 (18) is another strain producing CBP and cellobextrin phosphorylase (CDP; 1,4-β-D-oligoglucuron:orthophosphate α-D-glucosyl transferase, EC 2.4.1.49) intracellularly. The YM4 strain produces more cellulase-degrading activity than any other strains of Clost.

Cellobiose phosphorylase of Clostr. thermocellum described. It also has some physiological characteristics different from those reported for other strains in spore formation, nutrient requirement and carbohydrate utilization (19, 20). Since the YM4 strain was isolated, there has, however, been no report on genetic work or kinetic studies on CBP.

So far, four cellulobiose phosphorylase genes have been cloned (2, 7, 17), and it is known that there are many highly conserved regions in these enzymes. Comparison of the amino acid sequence of CBP from Clost. thermocellum ATCC27405 (AB013109) with those from Clost. Stercorarium (U56424), T. neapolitana (Z99777), and Cellv. gilvus (AB010707) revealed similarities of as high as 71, 73, and 62%, respectively. On the other hand, two CDPs have been cloned from Clost. thermocellum ATCC27405 (AB006822) and Clost. stercorarium (U60580), and there is only 23% similarity between the two enzymes. Recently, we cloned the cellobextrin phosphorylase gene of the Clost. thermocellum YM4 strain (21). It showed 92% similarity with that of Clost. thermocellum ATCC27405, promising that the CBPs of ATCC27405 and the YM4 strain would exhibit even higher similarity.

The reactions of cellulobiose phosphorylases from Cellvibrio gilvus (10, 15) and Cellulomonas uda (16) have been reported to follow the sequential bi bi mechanism, with the same order of substrate binding and product release: e.g. cellobiose binds to the enzyme before P, and then G 1-P is released after D-glucose. Maltose phosphorylase from Lactobacillus brevis (14) also follows the same reaction mechanism. Alexander (8) and Tanaka et al. (22) reported some
kinetic studies on cellulose phosphorylases from Clost.
thermocellum 653 and Clost. thermocellum ATCC27405.
However, no further kinetic studies or the reaction mecha-
nism of the enzyme has been reported, except that the cel-
lobiose phosphorylase gene of Clost. thermocellum ATCC-
27405 has been published in GenBank (AB013109).

We have been studying the phosphorolytic enzymes (CBP
cDP) of the Clost. thermocellum YM4 strain. In this
study, for a detailed understanding of the reaction mecha-
nism of CBP, the cbp gene of the Clost. thermocellum YM4
strain is closed using the PCR method and the recombi-
nant enzyme is purified after being expressed in Escheri-
chia coli. With the purified enzyme, the reaction mecha-
nisms of both the phosphorolytic and synthetic reactions
are extensively studied, and the substrate/acceptor specific-
ties and substrate inhibition by β-glucose are studied in
detail.

MATERIALS AND METHODS

Materials—α- and β-D-glucose, and d-cellobiose were
purchased from Sigma (St. Louis, USA). α-D-Glucose-1-
phosphate (G 1-P) dipotassium sulfate was purchased from
Nacalai Tesque (Kyoto). All other chemicals used here were
of reagent grade and used without any further purification.

Molecular Cloning of the Cbp Gene of the Clost.
thermocellum YM4 Strain—For PCR (polymerase chain reaction)
amplication of the cbp gene, the genomic DNA of the
Clost. thermocellum YM4 strain was purified with Insta-
Gene matrix (Biorad) and used as a template DNA. A pair of
primers (sense primer: 5′-CCATGGAGTTCGGTTTCATCT-
TGATGAT-3′, and anti sense primer: 5′-CTCGAGAAT-
TATTCAACTTTGTGAGTCTTT-3′) was designed based
on the DNA sequence of Clost. thermocellum ATCC27405
(AB013109). Underlining for the primers indicates the
restriction enzyme sites, Ncol and Xhol, respectively.
Through ligation via these restriction enzyme sites to the
same sites of the pET28a vector, a recombinant CBP har-
boring the 6xHis-tag at the C-terminus will be expressed in
E. coli.

PCR, 22 cycles, was performed using KOD-plus DNA
polymerase (Toyobo Biochemical, Osaka) under the follow-
ning conditions; 98°C, 1 min for denaturation, 55°C, 1 min
for annealing, and 68°C, 3 min for extension. The ampli-
fied PCR product was cloned into the pCR-XL-TOPO
vector using a TOPO XL PCR Cloning Kit (Invitrogen,
Carlsbad, CA, USA). More than three individual recombi-
nant plasmids were purified from the transformants and
the nucleotide sequences confirmed using a Big Dye Termi-
nator Cycle Sequencing Kit (Perkin-Elmer, Applied Biosys-
tems, Carlsbad, CA, USA). The confirmed recombinant plasmid
was then partially digested with Ncol and Xhol, and the target
expression system (15,000 x g, 15 min, 4°C), a Ni-NTA agarose slurry was
added to the supernatant to bind the CBP via a 6xHis tag.
The enzyme was then eluted with 150 mM imidazole in 50
mM phosphate buffer (pH 7.5) containing 1.4 mM 2-mer-
captoethanol. The resultant active fraction was loaded onto a Mono Q (Pharmacia)
column. The protein was eluted with 50 mM phosphate buffer
containing 1.4 mM 2-mercaptoethanol. The enzyme was eluted with a
linear gradient of 0 to 0.5 M NaCl in MOPS buffer (pH 7.5)
using an FPLC system (Pharmacia LKB Biotechnology).
The purity of the enzyme at each step was determined by
sodium dodecyl sulfate polyacrylamide gel electrophoresis
(SDS PAGE) and polyacrylamide gel electrophoresis
(Native PAGE) (23).

Assay Methods—The amount of protein was measured
based on the molar coefficient of A280 of 158200. G 1-P
was measured using the phosphoglucomutase-glucose-6-
phosphate dehydrogenase system (24). β-Glucose was measured
by the glucose oxidase peroxidase method with mutarotase
(25) using the Glucose CII Test (Wako Pure Chemicals,
Osaka). Quantification of P, in the presence of G 1-P
was carried out by the method of Lowry and Lopez (26).

Enzyme reactions were carried out at 37°C in 40 mM
MOPS buffer (pH 7.5) containing 1.4 mM 2-mercaptop-
ethanol. The initial rate of the phosphorolytic reaction was
determined by measuring the amounts of G 1-P and β-glu-
cose formed during the enzyme reaction with 10 mM cel-
lobiose and 10 mM phosphate (P). For that of the synthetic
reaction, the amounts of P, liberated from 10 mM G 1-P
and acceptors were determined.

Kinetic Parameters—To determine the apparent kinetic
parameters of the phosphorolytic reaction and synthetic
reactions, all the enzyme reactions were carried out under
the respective standard conditions unless otherwise speci-
fied. The reaction mixture containing substrates and en-
zyme (2.1 x 10−5 μmol), with a total volume of 500 μl, 
was incubated at 37°C. Samples (100 μl) were taken at intervals
and treated at 90°C for 10 min or mixed with sodium ace-
tate buffer (pH 3.6) to inactivate the enzyme used. The ini-
tial velocities were calculated from the linear relationship
of the product concentration against reaction time (0–30
min). The values for each parameter were calculated by
nonlinear regression analysis using a computer program,
“GraFit (Erithacus Software, Ver. 4.0).” The kinetic param-
ters of the sequential hi bi mechanism were calculated with
Eq. 1 (27):

\[ v = \frac{V_{\text{max}}[A][B]}{K_{\text{catA}}K_{\text{mB}} + K_{\text{mB}}[A] + K_{\text{mA}}[B] + [A][B]} \]  
\[ (A = P, B = \text{d-cellobiose}) \]

For the kinetic parameters of the substrate competitive in-
hibition by d-glucose, initial velocity was fitted to Eq. (2)
(10):

\[ v = \frac{V_{\text{max}}[P]}{K_{\text{catP}} + K_{\text{mP}}[P] + K_{\text{mQ}}[Q]} + \frac{K_{\text{mQ}}[Q]}{K_{\text{mQ}}[Q] + K_{\text{mP}}K_{\text{mQ}}[P] + K_{\text{mP}}[Q]} \]

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Reaction Mechanism of CBP

\[
+Q[P] + \left(K_{Q,K_{cat1}K_{cat2}} + K_{m2}[K_{cat1}]P\right) + K_{m2}[K_{cat1}][P]^3
\]

\[Q = G_1-P, P = glucose; \text{for detailed information, see Ref. 10}\]

RESULTS

Molecular Cloning of the Cbp Gene—With a set of primers that had been designed based on the DNA sequence of the cellobiose phosphorylase gene of *Clost. thermocellum* ATCC27405, a PCR product (2.4 kbp) was successfully amplified from the genomic DNA of the *Clost. thermocellum* YM4 strain. The PCR product (2.4 kbp) was cloned to the pCR-XL-TOPO vector and the nucleotide sequence was determined. Multiple alignment of the deduced amino acid sequence revealed that CBP from the YM4 strain (AY072794) exhibited 99.6% similarity with that of *Clost. ther-

Fig. 1. SDS and native PAGE of the recombinant CBP. SDS (A) and native PAGE (B) were performed by the methods of Laemmli (23) with 7.5 and 8% acrylamide, respectively. The protein on the gel was stained with Coomassie Brilliant Blue R-250. Protein markers (M), a 10 kDa ladder, were purchased from Pharmacia. M, protein markers; 1, the enzyme after Ni-NTA agarose purification; 2, after Superdex 200; 3, after Mono Q.

Fig. 2. $E_{\text{m}}$ vs. $1/[\text{cellulobiose}]$ plot of the phosphorolytic reaction with different concentrations of $P_i$. †, 0.8 mM $P_i$; †◦, 1.0 mM $P_i$; †, 1.5 mM $P_i$; †$, 2.0 mM P_i$; †¢, 2.5 mM $P_i$.

Fig. 3. Inhibition patterns of the products against the substrates. The initial concentrations of the other substrates were 0.8 mM $P_i$ (A, C) and 3 mM D-cellulobiose (B, D). □, G 1-P against CG2; ●, G 1-P against P_i; ○, glucose against CG2; ♦, glucose against P_i; ◊, none of initial product; ○, 0.5 mM G 1-P; ◊, 1.5 mM G 1-P; ○, 1 mM G 1-P; ○, 2 mM G 1-P; ◊, 2 mM glucose; ●, 4 mM glucose.
**mocellum ATCC27405.**

**Purification of the Recombinant CBP—**Purification of the CBP expressed in E. coli was carried out by three chromatographic methods, i.e. Ni-NTA agarose affinity chromatography, Superdex 200 gel filtration chromatography and Mono Q anion exchange chromatography (Fig. 1). After Mono Q chromatography, it emerged as a single band on SDS and Native PAGE. The molecular mass of the purified enzyme was estimated to be 92 kDa, which corresponded well to the M, (93,755) estimated from the deduced amino acid sequence. When the molecular weight of the purified enzyme was determined by gel permeation chromatography (Superdex 200), it was estimated to be 170 kDa, indicating that the enzyme is a homo-dimer in the native condition.

**Reaction Mechanism of CBP—**When double reciprocals of the initial velocities against the initial concentrations of D-cellulbiose were plotted at several fixed concentrations of P, the lines crossed at a certain point on the second quadrant (Fig. 2), indicating that the enzyme reaction follows the sequential bi bi mechanism (27). The inhibition patterns of the products, G 1-P and d-glucose, against the substrates, d-cellulbiose and P, are shown in Fig. 3. G 1-P acted as a competitive inhibitor against P, (Fig. 3B), whereas others (Fig. 3, A, C, and D) showed mixed type inhibition patterns. These patterns were confirmed by triplicate experiments. These results clearly indicate that the reaction of CBP of the Clost. thermocellum YM4 strain follows an ordered bi bi mechanism (27), in which P, binds to the enzyme before D-cellulbiose and then G 1-P is released after d-glucose (Scheme 1).

The kinetic parameters were calculated by regressing the data shown in Fig. 3 to Eq. (1) as follows; k, = 10.6 ± 0.5 (s⁻¹), K, = 0.49 ± 0.05 mM, K, = 0.81 ± 0.17 mM, and K, = 1.54 ± 0.51 mM (A, P; B, cellulbiose).

**Substrate Specificities in the Synthetic Reaction—**To understand the synthetic reaction, substrate specificities were investigated with 32 different compounds including pentoses, hexoses and their derivatives at 5 mM with 10 mM G 1-P (Table I). Among them, only eight (d-glucose, 6-deoxy-d-glucose, D-xylose, d-glucoaminde, D-mannose, 2-deoxy-d-glucose, 1,5-anhydro-d-glucitol, and d-glucuronamide) acted as acceptors. This specificity was similar to that of CBP from Clost. thermocellum 653 (8) and Cellu. gilvus (10), with the exception of the specificity to 1,5-anhydro-d-glucitol (Fig. 4). When the initial velocities for the substrates selected were investigated within the concentration range of 1–100 mM, all the substrates examined showed substrate inhibition patterns to some extent (Fig. 4). Among them, the strongest inhibition was observed with d-glucose. The apparent kinetic parameters (K, k, and k, = K, / K, were calculated at lower concentrations of each substrate where the inhibition was negligible (Table II).

**TABLE I. Substrate specificity in the synthetic reaction of CBP.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>v/E_p (s⁻¹)</th>
<th>Substrate</th>
<th>v/E_p (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>11.8</td>
<td>6-deoxy-D-glucose</td>
<td></td>
</tr>
<tr>
<td>D-Mannose</td>
<td>0.37</td>
<td>D-Glucose</td>
<td>26.5</td>
</tr>
<tr>
<td>2-Deoxy-d-glucose</td>
<td>1.46</td>
<td>D-Gluconic acid</td>
<td>3.76</td>
</tr>
<tr>
<td>D-Glucoaminde</td>
<td>0.37</td>
<td>d-Glucosamine</td>
<td>0.81</td>
</tr>
<tr>
<td>1,5-Anhydro-d-glucitol</td>
<td>0.67</td>
<td>d-Xylose</td>
<td>8.01</td>
</tr>
<tr>
<td>Methyl-β-d-glucoside</td>
<td></td>
<td>L-Isole</td>
<td></td>
</tr>
<tr>
<td>Methyl-α-d-glucoside</td>
<td></td>
<td>l-Arabinose</td>
<td></td>
</tr>
<tr>
<td>D-Mannitol</td>
<td></td>
<td>d-Fructose</td>
<td></td>
</tr>
<tr>
<td>L-Neu-Insolitol</td>
<td></td>
<td>D-Sorbitol</td>
<td></td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td></td>
<td>d-Galactose</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-d-glucosamine</td>
<td></td>
<td>d-Lyxose</td>
<td></td>
</tr>
<tr>
<td>3,6-Methyl-d-glucose</td>
<td></td>
<td>d-Ribose</td>
<td></td>
</tr>
<tr>
<td>1,6-Anhydro-d-glucose</td>
<td></td>
<td>d-Arabinose</td>
<td></td>
</tr>
</tbody>
</table>

For the reaction, 10 mM G 1-P and 5 mM each substrate were used. Minus (−) means under 2% of the D-Glucose value.

**Scheme I. Ordered kinetic mechanism of CBP from Clost. thermocellum YM4.**

\[
K_p \quad K_d \quad K_i \quad K_f \quad K_{f_2} \quad K_{f_3} \quad K_{f_4} \quad K_{f_5} \quad K_{f_6}
\]

E

E P

E G 1-P

E

To examine recognition of the α- and β-anomeric configurations of the acceptor molecule by the CBP, the reaction rates of these compounds, 1 mM, were investigated. In the early stage, the reaction rate of β-d-glucose is three times higher than that of α-d-glucose (Fig. 5), suggesting that the enzyme recognizes the β-anomeric hydroxyl group of d-glucose.

**Fig. 5. v[E_p] plots of the glucose acceptors.** α, 6-deoxy-D-glucose; •, d-xylose; o, d-glucose; ▼, D-glucoaminde; ▲, d-mannose; ◯, 2-deoxy-d-glucose; ⊙, 1,5-anhydro-d-glucitol; ●, d-glucuronamide. Solid lines are the calculated curves obtained with the Michaelis-Menten equation.

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TABLE II. Apparent kinetic parameters for the reaction of CBP with various substrate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_{\text{m}}$ (mM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_{\text{m}}/K_{\text{cat}}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxy-(\alpha)-glucose$^a$</td>
<td>6.1 ± 0.8</td>
<td>59.5 ± 3.5</td>
<td>9.750</td>
</tr>
<tr>
<td>(\beta)-Xylose$^b$</td>
<td>21.4 ± 1.3</td>
<td>44.3 ± 1.3</td>
<td>2.070</td>
</tr>
<tr>
<td>2-Deoxy-(\alpha)-glucose$^c$</td>
<td>56.9 ± 3.3</td>
<td>18.3 ± 0.7</td>
<td>322</td>
</tr>
<tr>
<td>(\beta)-Mannose$^d$</td>
<td>131 ± 20</td>
<td>9.5 ± 1.1</td>
<td>171</td>
</tr>
<tr>
<td>(\alpha)-Gluucose$^e$</td>
<td>8.9 ± 0.4</td>
<td>7.7 ± 0.2</td>
<td>131</td>
</tr>
<tr>
<td>1,5-Anhydro-(\beta)-glucitol$^f$</td>
<td>38.4 ± 2.1</td>
<td>7.3 ± 0.2</td>
<td>190</td>
</tr>
<tr>
<td>(\alpha)-Glucuronamid$^g$</td>
<td>20.3 ± 1.3</td>
<td>2.0 ± 0.1</td>
<td>99</td>
</tr>
<tr>
<td>(\alpha)-Glucose$^h$</td>
<td>0.97 ± 0.25</td>
<td>14.2 ± 2.2</td>
<td>14600</td>
</tr>
</tbody>
</table>

Values were calculated at the following concentrations: 1-10 mM, 5-40 mM, 5-50 mM, 15-70 mM, 1-15 mM, 5-40 mM, 5-20 mM, and 1-5 mM.

Inhibition Mechanism of \(\beta\)-Glucose—A remarkable decrease in the initial velocity of the reverse reaction was detected with an increase in the concentration of D-glucose at concentrations higher than 4 mM (Fig. 4). The rate with 100 mM D-glucose was only 15% of that with 4 mM. As there was remarkable inhibition by D-glucose, the inhibition pattern was investigated with five different concentrations of G 1-P. As shown in Fig. 6, the concentration of D-glucose giving the maximum velocity increased with an increase in the concentration of G 1-P, indicating that D-glucose acted as a competitive inhibitor. When the $K_{\text{m}}$ app and $k_{\text{cat}}$ app of G 1-P were plotted against the glucose concentration, $k_{\text{cat}}$ app showed a typical Michaelis Menten curve and $K_{\text{m}}$ app showed a parabolic curve (Fig. 7). These results suggest that the inhibition mechanism of CBP of the Clost. thermocellum YM4 strain follows the competitive substrate inhibition model mechanism proposed by Kitaoka et al. (10). Thus, the kinetic parameters were calculated based on Eq. (2). The kinetic parameters were calculated as follows: $k_{\text{cat}}$ = 11.4 ± 0.1 (s$^{-1}$), $K_{\text{cat}}$ = 3.74 ± 0.78 mM, $K_{\text{m}} = 0.11 ± 0.04$ mM, $K_{\text{m}} = 0.62 ± 0.06$ mM, $K_{\text{m}} = 2.75 ± 0.74$ mM, and $K_{\text{m}} = 10.4 ± 3.3$ mM (Q, G 1-P; P, glucose).

DISCUSSION

In this study, the cbp gene of the Clost. thermocellum YM4 strain (AY072794) was cloned by the PCR method. The amino acid sequence exhibited 99.6% similarity with that of Clost. thermocellum ATCC27405, indicating that these two cellobiose phosphorylases might have identical characters. However, on alignment, the sequence of CDP from the Clost. thermocellum YM4 strain exhibited 92% similarity to that of Clost. thermocellum ATCC27405 (21), indicating that the YM4 strain produced by CDP was different from that of Clost. thermocellum ATCC27405.

Disaccharide phosphorylases can be divided into two classes based on the anomeric configuration of the glucose-1-phosphate produced through the phosphorolytic reaction of the enzymes. For sucrose phosphorylase (28) the anomeric form is retained after phosphorolysis and for the others, such as cellobiose phosphorylase (1-8), maltose phosphorylase (29), trehalose phosphorylase (30), and laminaribiose phosphorylase (31), the anomeric form is inverted after phosphorylisis of the substrate. It was known that the reactions of all the inverting disaccharide phosphorylases so far reported proceed through an ordered bi bi mechanism. For instance, the reactions of CBPs from Cellu. gilvus (10-15) and Cellu. uda (16) proceed through an ordered bi bi mechanism, in which 1-cellobiose binds to the enzyme before P, and then G 1-P is dissociated from the E-
The Clost. thermocellum YM4 strain, in the case of CBP of the Clost. thermocellum YM4 strain, the reaction mechanism was not the same as for other CBPs (10, 15, 16), in which P_i bound to the enzyme before D-cellobiose and then the G 1-P was released after D-glucose (Scheme 1). It is interesting that the substrate binding order of CBP of Clost. thermocellum YM4 is different from that of Cellu. gilvus (10–15), although the amino acid sequence similarity is as high as 62%.

As shown in Table II, the biggest $k_{cat}/K_m$ was observed with D-glucose (14,639 M$^{-1}$s$^{-1}$), followed by 6-deoxy-D-glucose (9,754 M$^{-1}$s$^{-1}$) and D-xylose (2,070 M$^{-1}$s$^{-1}$). The apparent $K_m$ value of D-xylose (44.3 s$^{-1}$) was almost the same as that of 6-deoxy-D-glucose (58.5 s$^{-1}$), but the $K_m$ value of D-glucose (21.4 mM) was 3-times bigger than that of 6-deoxy-D-glucose (6.1 mM). This indicates that the methyl group of 6-deoxy-D-glucose plays a role only in binding with the enzyme. This finding is slightly different from the result for CBP from Cellu. gilvus (10), in which the methyl group of 6-deoxy-D-glucosyl plays a role in both binding and enzyme activity. When the C6 hydroxyl group was removed, as in the cases of 6-deoxy-D-glucose (59.5 s$^{-1}$) and D-xylose (44.3 s$^{-1}$), the $k_{cat}$ values increased to more than 3-times that of D-glucose (14.2 s$^{-1}$). This indicates that the C6 hydroxyl group plays an important role in the decrease in the $k_{cat}$ values. On the other hand, when the hydroxyl group of C2 was substituted, such as in the cases of 2-deoxy-D-glucose and D-mannose, the $k_{cat}$ values were not much changed compared to that of D-glucose, but there were 60- and 135-fold increases in the $K_m$ values, indicating that the hydroxyl group of C2 and its configuration are important for correct substrate binding. Nidetzky et al. (16) reported for CBP from Cellu. uda that the C2 hydroxyl group of the substrate binds to the enzyme by using a hydrogen bond and that the binding stabilizes the enzyme activity.

Kitaoka et al. (10) and Nidetzky et al. (16) reported that cellulose phosphorylases from Cellu. gilvus and Cellu. uda strictly recognize the equatorial anomeric hydroxyl group of D-glucose. No detectable synthetic activities of these enzymes were found with 1,5-anhydro-D-glucitol, the $k_{cat}$ values were 60 and 135-fold increases in the $K_m$ values, indicating that the hydroxyl group before D-cellobiose and then the G 1-P was released after D-glucose (Scheme 1). It is interesting that the substrate binding order of CBP of Clost. thermocellum YM4 is different from that of Cellu. gilvus (10–15), although the amino acid sequence similarity is as high as 62%.

In conclusion, the enzyme reaction of CBP from the Clost. thermocellum YM4 strain follows essentially the same mechanisms (sequential bi bi mechanism, substrate specificity and substrate inhibition) as other CBPs from Cellu. gilvus (10–15) and Cellu. uda (16), with the exception of the substrate specificity for 1,5-anhydro-D-glucitol. However, the substrate binding order is different, whereas the amino acid sequence similarity is very high (62%). However, it is still unclear what the difference in the order means. The formation of the binary complex (E–P) may have important roles as to the correct active-site conformation and enzyme activity of the CBP of the Clost. thermocellum YM4 strain.

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