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Kinetic Studies of a Recombinant Cellobiose Phosphorylase (CBP) of the *Clostridium thermocellum* YM4 Strain Expressed in *Escherichia coli*[1]

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A cellobiose 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 P_i 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 P_i. 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, cellobiose phosphorylase, *Clostridium thermocellum* YM4, competitive substrate inhibition, ordered bi bi mechanism.

Cellobiose phosphorylase (CBP; cellobiose:orthophosphate α-D-glucosyl transferase, EC 2.4.1.20) catalyzes the reversible phosphorolysis of D-cellobiose to α-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*).

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

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kinetic studies on cellobiose phosphorylases from *Clost. thermocellum* 653 and *Clost. thermocellum* ATCC27405. However, no further kinetic studies or the reaction mechanism of the enzyme has been reported, except that the cellobiose phosphorylase gene of *Clost. thermocellum* ATCC-27405 has been published in GenBank (AB013109).

We have been studying the phosphorolytic enzymes (CBP and CDP) of the *Clost. thermocellum* YM4 strain. In this study, for a detailed understanding of the reaction mechanism of CBP, the *cbp* gene of the *Clost. thermocellum* YM4 strain is cloned using the PCR method and the recombinant enzyme is purified after being expressed in *Escherichia coli*. With the purified enzyme, the reaction mechanisms of both the phosphorolytic and synthetic reactions are extensively studied, and the substrate/acceptor specificities and substrate inhibition by D-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) amplification 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′-CCATGGAGTTCGGTTTTTTT-TGATGAT-3′, and anti sense primer: 5′-CTCGAGAAT-TACTTCAACTTTGTGAGTCTTT-3′) was designed based on the DNA sequence of *Clost. thermocellum* ATCC27405 (AB013109). Underlining for the primers indicates the restriction enzyme sites, *Nco*I and *Xho*I, respectively. Through ligation *via* these restriction enzyme sites to the same sites of the pET28a vector, a recombinant CBP harboring 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 following conditions; 98°C, 1 min for denaturation, 55°C, 1 min for annealing, and 68°C, and 3 min for extension. The amplified 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 recombinant plasmids were purified from the transformants and the nucleotide sequences confirmed using a Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems, CA, USA). The confirmed recombinant plasmid was then partially digested with *Nco*I and *Xho*I, and the target fragment was ligated into the pET28a vector predigested with the same enzymes, and then the thus constructed vector (pET28-CBP) was transformed into *E. coli* BL21(DE3)-*p*LysS (Novagen, Madison, WI, USA) to yield an over-expression system.

Expression and Purification of the Recombinant CBP—For expression of the recombinant CBP, IPTG (isopropyl-β-D-thiogalactopyranoside) was added, when the A_{600} reached 0.6, to give a final concentration of 1 mM IPTG in LB-Kanamycin medium, and then the incubation was continued for a further 4 h. The *E. coli* cells were harvested by centrifugation (10,000 ×*g* for 5 min at 4°C), suspended in 50 mM phosphate buffer (pH 7.5) containing 1.4 mM 2-mercaptoethanol, 300 mM NaCl, and 20 mM imidazole, and then sonicated (Branson sonifier 250/450D) at 4°C to obtain a crude enzyme. After removing the cell debris by centrifugation (15,000 ×*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-mercaptoethanol, as described in the QIAexpressionist instructions (QIAGEN, Germany). The active fractions were pooled and then applied to a Superdex 200 (Pharmacia) column. The protein was eluted with 50 mM phosphate buffer containing 1.4 mM 2-mercaptoethanol. The resultant active fraction was loaded onto a Mono Q (Pharmacia) column equilibrated with 50 mM MOPS buffer (pH 7.5) 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 coefficiency of A_{280} of 158200. G 1-P was measured using the phosphoglucomutase-glucose-6-phosphate dehydrogenase system (*24*). D-Glucose was measured by the glucose oxidase peroxidase method with mutarotase (*25*) using the Glucose CII Test (Wako Pure Chemicals, Osaka). Quantification of P_i 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-mercaptoethanol. The initial rate of the phosphorolytic reaction was determined by measuring the amounts of G 1-P and D-glucose formed during the enzyme reaction with 10 mM cellobiose and 10 mM phosphate (P_i). For that of the synthetic reaction, the amounts of P_i 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 specified. The reaction mixture containing substrates and enzyme (2.1×10^{-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 acetate buffer (pH 3.6) to inactivate the enzyme used. The initial 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 parameters of the sequential bi bi mechanism were calculated with Eq. 1 (*27*):

$$v = V_{max}[A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (1)$$

(A = P_i, B = D-cellobiose)

For the kinetic parameters of the substrate competitive inhibition by D-glucose, initial velocity was fitted to Eq. (2) (*10*):

$$v = V_{max}[Q][P]/\{K_{iQ}K_{mP} + (K_{mQ} + K_{iQ}K_{mP}/K_{I1})[P] + K_{mP}[Q]$$

$$+[Q][P] + (K_{iQ}K_{mP}/K_{I1}K_{I2} + K_{mQ}/K_{I1})[P]^2 + K_{mQ}/K_{I1}K_{I2}[P]^3\} \quad (2)$$

(Q = G 1-P, P = glucose; 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 (AY-072794) exhibited 99.6% similarity with that of *Clost. ther-*

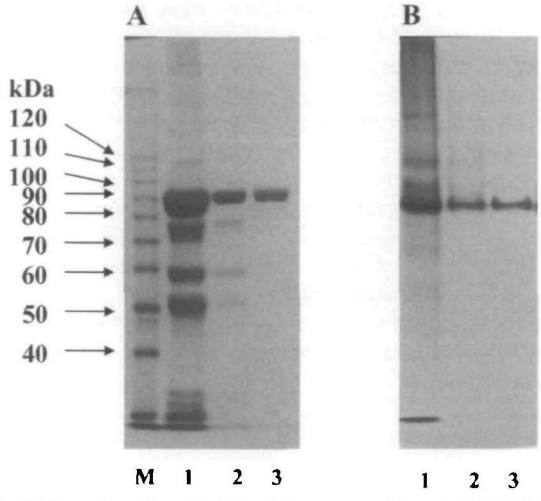


Fig. 1. **SDS and native PAGE of the recombinant CBP.** SDS (A) and native PAGE (B) were performed by the methods of Laemmli (*23*) with 7.5 and 8% acrylamide, respectively. The protein on the gel was stained with Coomassie Brilliant Blue R-250. Protein markers (M), a 10 kDa ladder, were purchased from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

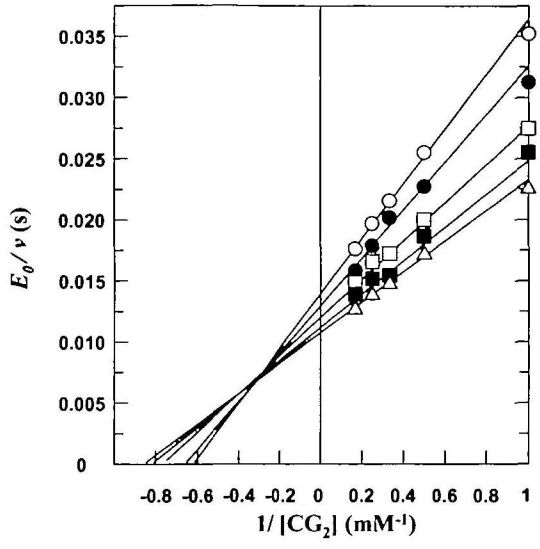


Fig. 2. **E_0/v–1/[cellobiose] plot of the phosphorolytic reaction with different concentrations of P_i.** ○, 0.8 mM P_i; ●, 1.0 mM P_i; □, 1.5 mM P_i; ■, 2.0 mM P_i; △, 2.5 mM P_i.

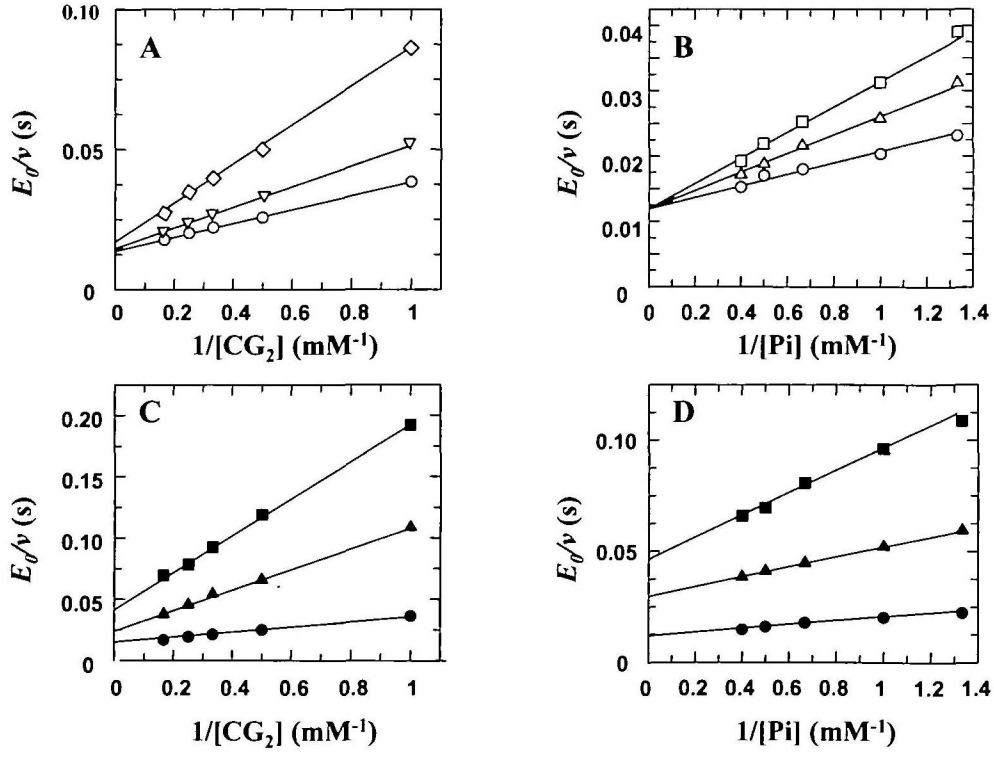


Fig. 3. **Inhibition patterns of the products against the substrates.** The initial concentrations of the other substrates were 0.8 mM P_i (A, C) and 3 mM D-celloboise (B, D). A, G 1-P against CG_2; B, G 1-P against P_i; C, glucose against CG_2; D, glucose against P_i; ○ and ●, none of initial product; ▽, 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.

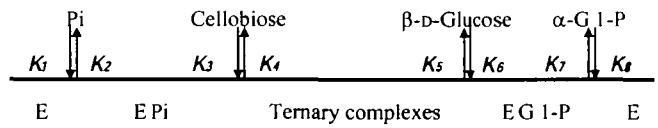
Purification of the Recombinant CBP—Purification of the CBP expressed in *E. coli* was carried out by three chromatographic methods, *i.e.* Ni-NTA agarose affinity chromatography, Superdex 200 gel filtration chromatography and Mono Q anion exchange chromatography (Fig. 1). After Mono Q chromatography, 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_r (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-cellobiose were plotted at several fixed concentrations of P_i, 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-cellobiose and P_i, are shown in Fig. 3. G 1-P acted as a competitive inhibitor against P_i (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_i binds to the enzyme before D-cellobiose 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_{cat} = 10.6 ± 0.5 (s^{-1}), K_{mA} = 0.40 ± 0.08 mM, K_{mB} = 0.81 ± 0.17 mM, and K_{iA} = 1.54 ± 0.51 mM (A, P_i; B, cellobiose).

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-glucosamine, 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 *Cellv. 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_m, k_{cat} and k_{cat}/K_m) were calculated at lower concentrations of each substrate where the inhibition was negligible (Table II).

To examine recognition of the α- and β-anomeric configurations of the acceptor molecule by the CBP, the reaction rates of α- and β-D-glucose, 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-glu-



Scheme 1. **Ordered kinetic mechanism of CBP from *Clost. thermocellum* YM4.**

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

Substrate	v/E_o (s^{-1})	Substrate	v/E_o (s^{-1})
D-Glucose	11.8	α-D-Glucose-1,6-diphosphate	–
D-Mannose	0.37	Glucose-6-phosphate	–
2-Deoxy-D-glucose	1.46	D-Glucuronic acid	–
D-Glucosamine	3.78	D-Glucono-δ-lactone	–
D-Xylose	8.01	L-Idose	–
6-Deoxy-D-glucose	26.5	L-Arabinose	–
D-Glucuronamide	0.37	L-Xylose	–
1,5-Anhydro-D-glucitol	0.57	L-Sorbose	–
Methyl-β-D-glucoside	–	L-Glucose	–
Methyl-α-D-glucoside	–	D-Allose	–
D-Mannitol	–	D-Fructose	–
myo-Inositol	–	D-Fucose	–
D-Sorbitol	–	D-Galactose	–
N-Acetyl-D-glucosamine	–	D-Lyxose	–
3-*O*-Methyl-D-glucose	–	D-Ribose	–
1,6-Anhydro-D-glucose	–	D-Arabinose	–

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

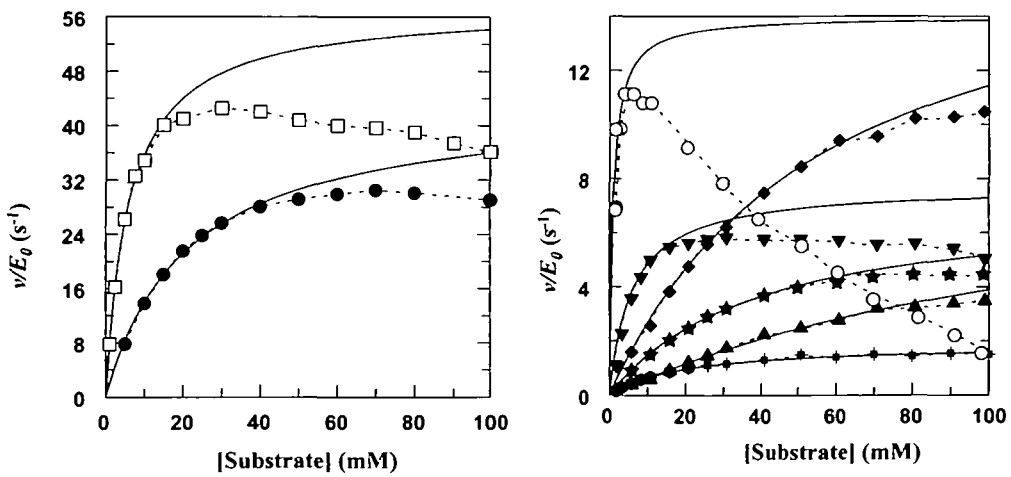


Fig. 4. **v/E_0–[s] plots of the glucosyl acceptors.** □, 6-deoxy-D-glucose; ●, D-xylose; ○, D-glucose; ▼, D-glucosamine; ▲, D-mannose; ◆, 2-deoxy-D-glucose; ★, 1,5-anhydro-D-glucitol; ■, D-glucuronamide. Solid lines are the calculated curves obtained with the Michaelis-Menten equation.

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

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
6-Deoxy-D-glucose[a]	6.1 ± 0.8	59.5 ± 3.5	9,750
D-Xylose[b]	21.4 ± 1.3	44.3 ± 1.3	2,070
2-Deoxy-D-glucose[c]	56.9 ± 3.3	18.3 ± 0.7	322
D-Mannose[d]	131 ± 20	9.3 ± 1.1	71
D-Glucosamine[e]	5.9 ± 0.4	7.7 ± 0.2	131
1,5-Anhydro-D-glucitol[f]	38.4 ± 2.1	7.3 ± 0.2	190
D-Glucuronamide[g]	20.3 ± 1.3	2.0 ± 0.1	99
D-Glucose[h]	0.97 ± 0.25	14.2 ± 2.2	14,600

Values were calculated at the following concentrations: [a]1–10 mM, [b]5–40 mM, [c]5–50 mM, [d]15–70 mM, [e]1–15 mM, [f]5–40 mM, [g]5–20 mM, and [h]1–5 mM.

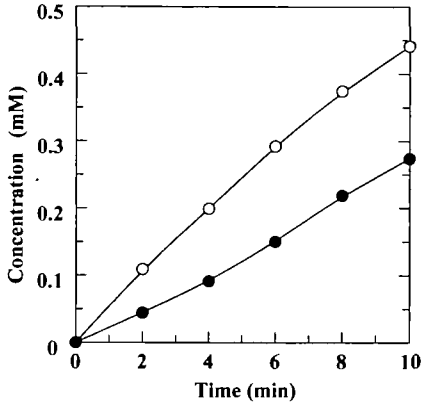


Fig. 5. **Reaction of cellobiose phosphorylase with α- and β-D-glucose.** ○, β-D-glucose; ●, α-D-glucose.

cose, if one exists.

Inhibition Mechanism of D-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_m app and k_{cat} app of G 1-P were plotted against the glucose concentration, k_{cat} app showed a typical Michaelis Menten curve and K_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_{cat} = 11.4 ± 0.1 (s^{-1}), K_{iQ} = 3.74 ± 0.78 mM, K_{mQ} = 0.11 ± 0.04 mM, K_{mP} = 0.62 ± 0.06 mM, K_{I1} = 2.75 ± 0.74 mM, and K_{I2} = 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

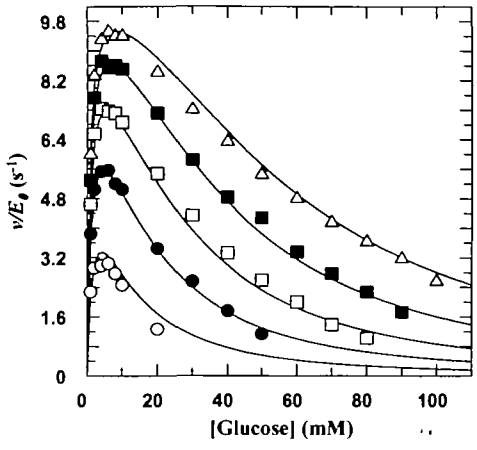


Fig. 6. **v/E_0–[Glucose] plot with various concentrations of G 1-P.** ○, 1 mM G 1-P; ●, 2.5 mM G 1-P; □, 5 mM G 1-P; ■, 10 mM G 1-P; △, 20 mM G 1-P.

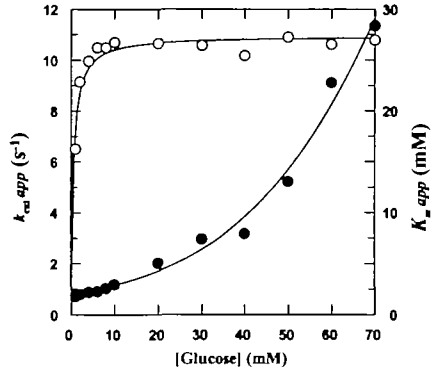


Fig. 7. **$k_{cat\ app}$– and $K_{m\ app}$–[Glucose] plot.** The $k_{cat\ app}$ and $K_{m\ app}$ values for G 1-P were calculated with the following equations: $k_{cat\ app} = V_{max}[P]/(K_{mP} + [P])[E]$ and $K_{m\ app} = (K_{iQ}K_{mP} + (K_{mQ} + K_{iQ}K_{mP}/K_{I1})[P] + K_{mP}[Q] + [Q][P] + (K_{iQ}K_{mP}/K_{I1}K_{I2} + K_{mQ}/K_{I1})[P]^2 + K_{mQ}/K_{I1}K_{I2}[P]^3)/(K_{mP} + [P])$. ○, $k_{cat\ app}$; ●, $K_{m\ app}$.

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 phosphorolysis 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 *Cellv. gilvus* (*10–15*) and *Cellu. uda* (*16*) proceed through an ordered bi bi mechanism, in which D-cellobiose binds to the enzyme before P_i and then G 1-P is dissociated from the E-

G 1-P intermediate in the last stage. 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 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 *Cellv. 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_{cat} 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-xylose (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 *Cellv. gilvus* (*10*), in which the methyl group of 6-deoxy-D-glucose plays a role in both binding and enzyme activity. When the C6 hydroxyl group was removed, such 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 cellobiose phosphorylases from *Cellv. 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 structure of which was described as that of 1-deoxyglucose. Using CBP from the YM4 strain, we found similar anomeric specificity (3 times higher activity with β-D-glucose than α-D-glucose) except that 1,5-anhydro-D-glucitol was active as an acceptor. The K_m and k_{cat} values were 38.4 mM and 7.3 s^{-1}, respectively. This indicates that CBP of the YM4 strain recognizes the β-hydroxyl group of D-glucose, but that the hydroxyl group is not essential for the enzyme activity.

Inhibition by D-glucose has been reported for several phosphorolytic enzymes such as phosphorylase (*32*), sucrose phosphorylase (*28*), cellobiose phosphorylase (*10*), and laminaribiose phosphorylase (*33*). In the cases of phosphorylase and sucrose phosphorylase, D-glucose only acts as an inhibitor. However, in the case of CBP from *Cellv. gilvus*, it acts not only as a substrate but also as an inhibitor. Kitaoka *et al.* (*10*) studied the inhibition mechanism of CBP from *Cellv. gilvus* by means of kinetic analyses, and reported that the inhibition was caused by competition for the G 1-P binding site by D-glucose, so called competitive substrate inhibition. The inhibition of CBP from the *Clost. thermocellum* YM4 strain seemed identical with that of *Cellv. gilvus*. The K_{mQ} (0.11 ± 0.03 mM) and K_{mP} (0.62 ± 0.06 mM) calculated using Eq. 2 were one-twentieth and one-fourth those of CBP from *Cellv. gilvus* (*10*), respectively. This indicates that there are notable differences in their substrate affinities.

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 *Cellv. 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_i) 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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