Enzymatic Characteristics of Cellobiose Phosphorylase from *Ruminococcus albus* NE1 and Kinetic Mechanism of Unusual Substrate Inhibition in Reverse Phosphorolysis

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Cellulose phosphorylase (CBP) catalyzes the reversible phosphorylorysis of cellobiose to produce α-β-glucopyranosyl phosphate (Glc1P) and β-glucose. It is an essential enzyme for the metabolism of cello-oligosaccharides in a ruminal bacterium, *Ruminococcus albus*. In this study, recombinant *R. albus* CBP (RaCBP) produced in *Escherichia coli* was characterized. It showed highest activity at pH 6.2 at 50 °C, and was stable in a pH range of 5.5–8.8 and at below 40 °C. It phosphorylized only cellobiose efficiently, and the reaction proceeded through a random-ordered bi bi mechanism, by which inorganic phosphate and cellobiose bind in random order and β-glucose is released before Glc1P. In the synthetic reaction, RaCBP showed highest activity to β-glucose, followed by 6-deoxy-α-glucose, α-Mannose, 2-deoxy-β-glucose, α-glucosamine, β-xyllose, 1,5-anhydro-α-glucitol, and gentiobiose also served as acceptors, although the activities for them were much lower than for β-glucose. α-Glucose acted as a competitive-uncompetitive inhibitor of the reverse synthetic reaction, which bound not only the Glc1P site (competitive) but also the ternary enzyme-Glc1P-β-glucose complex (uncompetitive).

Key words: cellobiose phosphorylase; substrate inhibition; substrate specificity; *Ruminococcus albus*

Obligatory anaerobic bacteria in the rumen produce many cellulolytic enzymes to degrade dietary cellulose, and the resulting digest is utilized by ruminant animals such as sheep and cows.† Cello-oligosaccharides are predominant end-products of the degradation of cellulose, and are crucial carbon sources for the growth of these bacteria.‡,§

*Ruminococcus albus* is a typical ruminal bacterium that produces extracellular cellulolytic enzymes.¶–‖ It metabolizes cello-oligosaccharides through phosphorolysis, catalyzed by cellobiose phosphorylase (CBP) and celloolactosyl phosphorylase, as well as hydrolysis by β-glucosidase.¶–‖ The phosphorolysis of cello-oligosaccharides is believed to be a more important process for *R. albus*, because intracellular phosphorolysis activity is considerably more intense than hydrolysis,§ but the biochemical characteristics of these carbohydrate phosphorolysases have not been investigated thus far.

CBP, which is known to be present in several microorganisms, e.g., *Cellvibrio gilvus*,††,‡‡ *Clostridium septicum*,†† *Ruminococcus flavefaciens*,†† *Cellulomonas uda*,†† *Forms annosus*,,* Thermotoga neapolitana*,,* Clostridium thermocellum*,,* and *Thermotoga maritima*,§ catalyzes the reversible phosphorolysis of cellobiose to produce α-β-glucopyranosyl phosphate (Glc1P) and β-glucose. In the phosphorolysis of cellobiose, the order of product release is common to all the CBPs kinetically analyzed (β-glucose and Glc1P are released in this order),¶–‖ In contrast, the order of substrate binding differs depending on the enzymes involved.¶–‖ The CBPs from *C. gilvus* and *C. uda* bind cellobiose before phosphate,¶–‖ whereas *C. thermocellum* CBP binds substrates in the inverse order.¶–‖ *T. maritima* CBP, however, was found to bind substrates in random order.¶–‖

The carbohydrate-active enzymes involved in the formation and cleavage of glycosidic linkages are categorized into three classes, glycoside hydrolases (GHs),‖ glycosyl transferases (GTs),‖ and polysaccharide lyases.‖ Each class comprises families classified on the basis of similarity of amino acid sequence. Carbohydrate phosphorylases are found in both the GT and the GH classes. The carbohydrate phosphorylases of the GT families have structures and catalytic mechanisms similar to GTs. For example, glycogen phosphorylase, which belongs to GT family 35, is formed by a GT-B fold‖ and shows a strong resemblance in its active site structure to typical GTs such as trehalose 6-phosphate synthase (OtsA)‖ and glycon synthase,‖ classified into GT families 20 and 5 respectively. On the other hand, GH-type carbohydrate phosphorylases are classified into GH families 13, 65, 94, and 112, and show significant similarity to glycoside hydrolases in structure and catalytic mechanism.‖

CBP was first classified into GT family 36 together with chitobiose phosphorylase, celloolactosyl phosphorylase, and cyclic β-1,2-glucan synthase. But three-dimensional structural analysis of *Vibrio proteolyticus* chitobiose phosphorylase revealed that it has a catalytic domain fold very similar to that of glucoamylase, a

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Abbreviations: CBP, cellobiose phosphorylase; RaCBP, CBP from *Ruminococcus albus* NE1; Glc1P, α-β-glucopyranosyl phosphate; GH, glycoside hydrolase; GT, glycosyl transferase
typical inverting glycosidase.\textsuperscript{31} Hence these enzymes were re-classified into GH family 94. Their reaction mechanism is believed to be analogous to those of inverting glycosidases (Fig. 1).\textsuperscript{31,32} The general acid catalyst donates a proton to the glycosidic oxygen, like inverting glycosidases. Simultaneously, the catalytic phosphate nucleophilically attacks the anomeric carbon of glycosyl residue at subsite $-1$ to give a product with an inverted anomeric configuration.

Complete genome sequence analysis of \textit{R. albus} 7 (GenBank ID, CP002403) revealed that the Rumal\textsubscript{0187} and Rumal\textsubscript{2403} proteins are putative GH family 94 enzymes. The Rumal\textsubscript{0187} protein shows higher similarity to known CBPs than the Rumal\textsubscript{2403} protein. Its sequence identities to CBPs from \textit{C. thermocellum}, \textit{C. giilus}, \textit{T. maritima}, and \textit{C. uda} are 67, 55, 62, and 53\% respectively. In this study, we cloned the CBP gene from \textit{R. albus} NE1, which was isolated from the cow rumen and is phylogenetically very close to \textit{R. albus} 7 (the 16S rDNA sequence of \textit{R. albus} NE1 is 99.86\% identical to that of \textit{R. albus} 7),\textsuperscript{34} and the recombinant enzyme produced in \textit{Escherichia coli} was characterized.

Materials and Methods

\textbf{Materials.} Cellobiose, D-glucose, D-xylose, methyl a-D-glucoside, methyl beta-D-glucoside, D-mannitol, D-glucitol, N-acetyl-D-glucosamine, D-glucose 6-phosphate, l-arabinose, D-fructose, D-galactose, lactose, and laminaribiose were purchased from Nacalai Tesque (Kyoto, Japan). 2-Decoxy-D-glucose, D-glucosamine, D-glucorarnid, and 1,6-anhydro-D-glucose were from Tokyo Chemical Industries (Tokyo). 1,5-Anhydro-D-glucitol, 3-O-methyl-D-glucose, and gentiobiose were from Wako (Osaka, Japan). D-Mannose, myo-inositol, and D-glucono-\delta-lactone were from Seikagaku (Tokyo). 6-Deoxy-D-glucose and chitin were from Sigma (St. Louis, MO).

\textbf{Cloning of the RaCBP gene and construction of the expression plasmid.} The RaCBP gene was obtained by PCR. The genomic DNA of \textit{R. albus} NE1, prepared as described previously,\textsuperscript{33} was used as template. A set of primers, 5'-ATAGGATCCCTCCCGGTT-3' (sense) and 5'-CGTGGATCCATGATACATCCATACA-3' (antisense), were designed on the basis of the sequence of the Rumal\textsubscript{0187} gene from \textit{R. albus} 7. Primestar HS DNA polymerase (Takara Bio, Ohtsu, Japan), 2-Decoxy-D-glucose, D-glucosamine, and gentiobiose were from Wako (Osaka, Japan). D-Mannose, myo-inositol, and D-glucosamine were from Sigma (St. Louis, MO). 1,6-Anhydro-D-glucose were from Seikagaku (Tokyo). 6-Deoxy-D-glucose and sophorose were from Sigma (St. Louis, MO).

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig1.png}
\caption{Reaction Mechanism of Cellobiose Phosphorylase. R indicates D-glucose moiety.}
\end{figure}

\textbf{Production and purification of recombinant RaCBP.} \textit{E. coli} BL21 (DE3) was transformed with the expression plasmid by Hanahan’s method.\textsuperscript{36} The transformant was propagated at 37° C in 500 mL of Luria-Bertani broth containing 50 \mu g/mL of ampicillin until A\textsubscript{600} reached 0.6. Production of recombinant RaCBP was induced by the addition of 0.1 mM isopropyl beta-D-thiogalactoside (final concentration, 0.1 mM), and incubation was continued at 30° C for a further 6 h with vigorous shaking.

\textit{E. coli} cells were harvested by centrifugation and resuspended in 20 mL of 20 mM MES-NaOH buffer (pH 7.0) containing 0.5 mM NaCl (buffer A). The bacterial cells were disrupted by sonication using Sonifier 450 (Branson, Danbury, CT), and cell debris was removed by centrifugation. The resulting supernatant was applied to a Ni-chelating Sepharose column (61.5 x 10 cm; GE Healthcare) equilibrated with buffer A. After thorough washing with buffer A containing 30 mM imidazole, the adsorbed protein was eluted with buffer A containing 0.5 mM imidazole. The purity of the enzyme preparation was confirmed by SDS-PAGE following Laemmli,\textsuperscript{37} and highly purified fractions were pooled. The collected fractions were dialyzed against 20 mM MES-NaOH buffer (pH 6.0) and frozen at −80° C for storage.

\textbf{Protein assay.} The protein concentrations of the crude extract and fractions in column chromatography were determined by the Bradford method\textsuperscript{38} and the UV method\textsuperscript{39} respectively. Bovine serum albumin (Nacalai Tesque) was used as standard protein for the Bradford method. In the UV method, the protein concentration was calculated based on an extinction coefficient of 0.1% w/v of the purified RaCBP, 2.35. The concentration of the purified enzyme was calculated based on the concentrations of the various amino acids after acid hydrolysis. The sample was incubated in 6 N HCl at 110°C for 24 h, and concentrations of the resulting amino acids were determined by the ninhydrin colorimetric method using JLC-500V (Jeol, Tokyo).\textsuperscript{40}

\textbf{Standard enzyme activity assay.} D-Glucose liberated from cellobiose by phosphorylase was measured by the glucose oxidase-peroxidase method.\textsuperscript{41} A reaction mixture of 100 \mu L containing an appropriate concentration of the enzyme, 10 mM cellobiose, and 10 mM sodium phosphate buffer (pH 6.0) was incubated at 37°C for 10 min. The enzyme was diluted with 20 mM MES-NaOH buffer (pH 6.0) containing 1 mg/mL of bovine serum albumin. The enzyme reaction was stopped by adding 50 \mu L of 4 mM Tris–HCl (pH 7.0). Tris inhibited the CBP reaction, and the reaction was stopped completely under these conditions. The liberated D-glucose was measured by Glucose CII Test (Wako). One U of enzyme activity was defined as the amount of enzyme producing 1 \mu mol of D-glucose from cellobiose in 1 min under these conditions.

\textbf{Optimum pH and temperature.} The optimum pH for the phosphorylase of cellobiose was investigated by measuring reaction velocities at various pH values. A reaction mixture of 100 \mu L consisting of 1.7 mM RaCBP, 10 mM cellobiose, 5 mM phosphate, and 250 mM of reaction buffer (sodium citrate buffer for pH 3.4–6.2, MES-NaOH buffer for pH 6.2–6.9, and Tris–HCl buffer for pH 6.9–8.7) was incubated at
37 °C for 10 min. The amount of d-glucose liberated was measured as described above. The optimum temperature for phosphorylation was evaluated by measuring the activity at 25–60 °C. The other reaction conditions were same as for the standard assay method.

**pH and temperature stabilities.** To determine a stable range of pH, residual activity was measured after pH treatment. An enzyme solution consisting of 2.1 μM RaCBP and 20 mM sodium citrate buffer for pH 3.0–6.1, MES-NaOH buffer for pH 6.0–7.0, Tris–HCl buffer for pH 6.8–8.7, and glycine-NaOH buffer for pH 8.8–10.3 was incubated at 4 °C for 24 h. Residual activities were measured after dilution of the sample, as described above. Thermal stability was evaluated based on residual activity after heat treatment. RaCBP of 2.9 nM was incubated in 20 mM MES- NaOH buffer (pH 6.0) at 25–60 °C for 20 min. The ranges of pH and temperature in which the enzyme retained more than 90% of original activity were considered to be stable ranges.

**Substrate specificity.** The substrate specificity for phosphorylation was investigated by measuring the initial velocities for 10 mM of the following oligosaccharides: cellobiose, laminaribiose, sophorose, gentiobiose, and lactose. The enzyme concentrations were 1.7 nM and 860 nM for cellobiose and the other substrates respectively. The other substrates were screened with the 24 carbohydrates listed in Table 1 by measuring initial velocities for 10 mM of the following oligosaccharides: cellobiose, and 0.8–2.5 mM sodium phosphate buffer (pH 6.0) was added to 2.9 nM MES- NaOH buffer (pH 6.0) at 25–60 °C for 20 min. The ranges of pH and temperature in which the enzyme retained more than 90% of original activity were considered to be stable ranges.

Acceptor substrates were screened with the 24 carbohydrates listed in Table 1 by measuring initial velocities for the release of inorganic phosphate. A reaction mixture of 50 μL consisting of RaCBP, 10 mM Glc1P, 10 mM acceptor substrate, and 20 mM MES-NaOH buffer (pH 6.0) was incubated at 37 °C for 10 min. The enzyme concentration was 8.6 mM for d-glucose and 6-deoxy-d-glucose, and 86 nM for the other substrates. The reaction was terminated by adding 50 μL of 4 mM Tris–HCl buffer (pH 7.0), and the inorganic phosphate released was measured following Lowry and Lopez. To determine the apparent kinetic parameters, the initial velocities at various concentrations of acceptor substrates and 10 mM Glc1P were measured and fitted to the Michaelis-Menten equation.

**Analysis of the kinetic mechanism of the phosphorolytic reaction.** To determine the kinetic parameters for the phosphorolytic activity of cellobiose, the initial reaction velocities were measured at various concentrations of cellobiose and sodium phosphate buffer (pH 6.0). A reaction mixture of 100 μL consisting of 1.7 mM RaCBP, 1–5 mM cellobiose, and 0.8–2.5 mM sodium phosphate buffer (pH 6.0) was incubated at 37 °C for 10 min, and d-glucose produced was measured as described above. The kinetic parameters were determined by fitting the reaction rates to the equation for the sequential bi bi mechanism:37

\[
v = \frac{k_{cat}[A][B]}{K_{d1}K_{d2} + K_{d1}[A]} + \frac{K_{d1}[A]}{[A][B]}
\]

(A = cellobiose, B = inorganic phosphate) (1)

The orders of substrate binding and product release through a sequential reaction were determined by product inhibition analysis. First, the initial reaction rates at varying concentrations of cellobiose (1–5 mM) and 10 mM sodium phosphate buffer (pH 6.0) were measured in the presence of 0–2 mM d-glucose and 0–1 mM Glc1P. Then the initial reaction rates at varying concentrations (1–5 mM) of sodium phosphate buffer (pH 6.0) and 5 mM cellobiose were measured in the presence of glucose and of Glc1P, as described above. In inhibition analysis by d-glucose, produced Glc1P was measured by the phosphoglucomutase-glucose-6-phosphate dehydrogenase method.38

**Analysis of the kinetic mechanism of the synthetic reaction.** Initial reaction rates at varying concentrations of d-glucose and Glc1P were measured. A reaction mixture of 100 μL consisting of 2.1 mM RaCBP, 2–300 mM d-glucose, 0.25–6 mM Glc1P, and 20 mM MES-NaOH buffer (pH 6.0) was incubated for 10 min, and the inorganic phosphate produced was measured as described above.

\[
E + Q \xrightleftharpoons[k_{-1}]{k_{1}} EQ + P \xrightleftharpoons[k_{-2}]{k_{2}} EPQ \xrightleftharpoons[k_{-3}]{k_{3}} E + A + B
\]

\[
P \xrightleftharpoons[k_{i1}]{k_{1}} P + P \xrightleftharpoons[k_{i2}]{k_{2}} EP + P \xrightleftharpoons[k_{i3}]{k_{3}} EPQ
\]

**Fig. 2.** Kinetic Scheme for the Synthetic Reaction of d-Glucose and Glc1P.

A, B, P, and Q indicate cellobiose, inorganic phosphate, d-glucose, and Glc1P respectively.

In an inhibition model of the second substrate (d-glucose) in a competitive-uncompetitive manner (Fig. 2), the reaction rate is given by the following equation:

\[
v = \frac{k_{cat}[P][Q]/[K_{d2}K_{d1} + K_{d2}]}{+ (K_{d2} + K_{d2}K_{d1} + [P])} + (K_{d2} + K_{d2}K_{d1} + [P] + Q/[K_{d2}])^2 + K_{d2}/[K_{d2}^2(K_P)]
\]

(2)

where the steady state kinetic parameters were as follows: $K_d = k_{-1}/k_{1}$, $K_{d2} = (k_{-2} + k_{1})$, $K_{d1} = k_{-1} + k_{1}$, $K_{d3} = k_{-3}/k_{1}$, $K_{d4} = k_{-4}/k_{1}$, and $K_{d5} = k_{-5}/k_{1}$ (P = d-glucose, Q = Glc1P). At constant [P], the reaction rates at various [Q] obey a Michaelis-Menten curve, and the apparent kinetic parameters for Glc1P, $k_{cat}^{app}$ and $K_m^{app}$, are given by the following equations:

\[
k_{cat}^{app} = k_{cat}[P]/(K_m + [P])^2/K_1
\]

(3)

\[
K_m^{app} = (K_{d2}K_{d1} + K_{d2} + K_{d2}K_{d1} + [P])^2 + K_{d2}/[K_{d2}^2(K_P)]
\]

(4)

To determine the kinetic parameters for the synthetic reaction to glucose and Glc1P, the apparent kinetic parameters, $k_{cat}^{app}$ and $K_m^{app}$, for Glc1P at varying concentrations of d-glucose were determined. The $k_{cat}^{app}$ values at various [P] were fitted to eq. (3), and $k_{cat}$, $K_m$, and $K_1$ were determined. Then the $K_m^{app}$ values at various [P] were fitted to eq. (4) to determine $K_{d2}$, $K_{d1}$, and $K_{d5}$ ($k_{cat}$, $K_m$, and $K_1$ obtained were assigned to eq. (4)).

**Results**

**Cloning and production of recombinant RaCBP in E. coli**

The RaCBP gene was obtained from the genomic DNA of *R. albus* NE1 by PCR. Primers designed based on the sequence of the Rumal_0187 gene were used. The sequence of the amplified DNA fragment was entirely identical to that of the Rumal_0187 gene. The cloned RaCBP gene was overexpressed in *E. coli* BL21 (DE3) to produce a recombinant enzyme harboring the His-Tag at the C-terminal. Recombinant RaCBP was purified to homogeneity by Ni-chelating column chromatography. Twenty mg of the purified enzyme, the specific activity of which was 60.5 U/mg, was obtained from 250 mL of the culture fluid. The purified enzyme gave a single band of 94 kDa on SDS–PAGE, coinciding with the theoretical molecular mass. Its molecular mass measured by gel filtration column chromatography was 187 kDa, indicating that RaCBP formed a homo-dimer in solution, like known CBPs.17,19,20 It showed maximum activity at pH 6.2 at 50 °C, and was stable in a range of pH 5.5–8.8 at below 40 °C. 

\[
\begin{align*}
E + Q & \xrightleftharpoons[k_{-1}]{k_{1}} EQ + P \\
& \xrightleftharpoons[k_{-2}]{k_{2}} EPQ \\
& \xrightleftharpoons[k_{-3}]{k_{3}} E + A + B
\end{align*}
\]
Cellobiose Phosphorylase from Ruminococcus albus

Table 1. Substrate Specificity for Reverse Synthetic Reaction of RaCP

<table>
<thead>
<tr>
<th>Substrate</th>
<th>v (s⁻¹)</th>
<th>Relative v (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>76.9 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>D-Mannoose</td>
<td>0.850 ± 0.05</td>
<td>1.11</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>3.66 ± 0.13</td>
<td>4.76</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>3.40 ± 0.10</td>
<td>4.42</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>3-O-Methyl-D-glucose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>6-Deoxy-D-glucose</td>
<td>48.7 ± 0.2</td>
<td>63.3</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>3.70 ± 0.05</td>
<td>4.81</td>
</tr>
<tr>
<td>D-Glucose 6-phosphate</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>D-Glucuronamide</td>
<td>trace</td>
<td>N. D.</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>1,2-Anhydro-D-glucitol</td>
<td>1.38 ± 0.05</td>
<td>1.79</td>
</tr>
<tr>
<td>1,6-Anhydro-D-glucose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>D-Glucitol</td>
<td>trace</td>
<td>N. D.</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>D-Glucono-β-lactone</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Methyl α-D-glucoside</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Methyl β-D-glucoside</td>
<td>trace</td>
<td>N. D.</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Sophorose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>1.52 ± 0.15</td>
<td>1.98</td>
</tr>
</tbody>
</table>

Initial velocity for 10 mM of Glc1P and each acceptor substrate was measured. Data are mean ± SD for three independent experiments. Trace, 0.5–1% of velocity for glucose. N. D., not determined.

Substrate specificity

Specificity for phosphorolysis was investigated by measuring the initial reaction rates for various disaccharides, cellobiose, laminaribiose, sophorose, gentiobiose, and lactose. RaCBP efficiently phosphorylated only cellobiose, and showed weak but detectable activity toward lactose. The other oligosaccharides tested were not phosphorylated. The apparent kinetic parameters, $k_{cat \text{ app}}$ and $K_{m \text{ app}}$, were measured in the presence of 10 mM sodium phosphate buffer (pH 6.0). $k_{cat \text{ app}}$ and $K_{m \text{ app}}$ for cellobiose were 79.1 ± 5.2 s⁻¹ and 0.11 mM respectively, and those for lactose were 0.0574 ± 0.011 s⁻¹ and 33.2 ± 13.6 mM respectively.

In the reverse synthetic reaction in the presence of 10 mM Glc1P, RaCBP showed measurable activities to eight sugars, D-glucose, D-mannose, 2-deoxy-D-glucose, D-glucosamine, D-xylose, 6-deoxy-D-glucose, 1,5-anhydro-D-glucitol, and gentiobiose (Table 1). The activities to D-glucuronamide, D-sorbitol, and methyl β-D-glucoside were detectable, but too low to be determined (less than 1% of activity relative to D-glucose). Inorganic phosphate released was detected in the reactions toward 3-O-methyl-D-glucose and laminaribiose, but high performance liquid chromatography analysis revealed that the reaction product was cellobiose, which was provably produced from contaminated D-glucose in the substrates used (data not shown). The apparent kinetic parameters for the eight sugars were measured in the presence of 10 mM Glc1P (Table 2). D-Glucose showed the highest $k_{cat \text{ app}}$ (92.3 ± 1.2 s⁻¹) and the lowest $K_{m \text{ app}}$ (1.46 ± 0.08 mM) among the substrates tested, followed by 6-deoxy-D-glucose, in terms of apparent catalytic efficiency. The $k_{cat \text{ app}}$ values for 2-deoxy-D-glucose and D-xylose were relatively high, but the $K_{m \text{ app}}$ values for them were much higher than D-glucose, resulting in considerably lower apparent catalytic efficiency.

Kinetic mechanism of phosphorolysis for cellobiose

Reaction rates at varying concentrations of cellobiose and phosphate were measured. The lines, obtained from double reciprocals plots for 1/v versus 1/cellobiose at various concentrations of inorganic phosphate, crossed at a certain point in the upper left quadrant, indicating that the phosphorolytic reaction occurred through a sequential bi bi mechanism (Fig. 3). The kinetic parameters were calculated as follows: $k_{cat} = 110 ± 6\, \text{s}^{-1}$, $K_{max} = 1.27 ± 0.02\, \text{mM}$, $K_{phosphate} = 0.427 ± 0.006\, \text{mM}$, $K_{A} = 8.93 ± 1.93\, \text{mM}$ (A, cellobiose; B, inorganic phosphate).

The inhibition patterns of the reaction products, Glc1P and D-glucose, against the phosphorolysis of cellobiose at various concentrations of cellobiose and inorganic phosphate are summarized in Fig. 4. Glc1P acted as a competitive inhibitor against both cellobiose and inorganic phosphate (Fig. 4a and b), whereas D-glucose showed mixed-type inhibition (Fig. 4c and d). This clearly indicates that phosphorolysis by RaCBP obeys a random-ordered bi bi mechanism, by which inorganic phosphate and cellobiose bind in a random order, and D-glucose is released before Glc1P.
Fig. 4. Product Inhibition for the Phosphorylysis of Cellobiose.
The concentrations of fixed substrates were 10 mM inorganic phosphate for (a) and (c) and 5 mM cellobiose for (b) and (d). a and b, Hollow circle, square, and triangle indicate 1, 0.5, and 0 mM Glc1P respectively. c and d, Solid circle, square, and triangle indicate 2, 1, and 0 mM D-glucose respectively.

Kinetic mechanism of reverse synthetic reaction for D-glucose and Glc1P

In the reverse synthetic reaction to D-glucose and Glc1P, a significant decrease in the initial velocity was observed at higher than 20 mM of D-glucose (Fig. 5a), although substrate inhibition by Glc1P was not observed under the conditions analyzed (Fig. 5b). The apparent kinetic parameters for Glc1P, $k_{\text{cat app}}$ and $K_{m \text{ app}}$, were calculated from the reaction rates at given D-glucose concentrations. $k_{\text{cat app}}$ decreased at high D-glucose concentrations, unlike the other CBPs reported.\textsuperscript{20,45} The apparent kinetic parameters fitted well to the theoretical lines obtained from eq. (3) and eq. (4) (Fig. 5c). This strongly confirms the proposed model shown in Fig. 2. The kinetic parameters, calculated by fitting the apparent kinetic parameters to eq. (3) and eq. (4), were as follows: $k_{\text{cat}} = 130 \pm 3$ s\(^{-1}\), $K_{dQ} = 0.306 \pm 0.079$ mM, $K_{mb} = 2.13 \pm 0.14$ mM, $K_{mq} = 0.00340 \pm 0.00020$ mM, $K_{i1} = 5.85 \pm 1.95$ mM, $K_{i2} = 28.2 \pm 10.7$ mM, and $K_{i3} = 398 \pm 99$ mM (P, D-glucose; Q, Glc1P). The lines obtained from the calculated kinetic parameters fitted well to the experimental values, as shown in Fig. 5a.

Discussion

In \textit{R. albus}, CBP is known to play a crucial role in the metabolism of cello-oligosaccharides,\textsuperscript{9} but its enzymatic characteristics have not been investigated. We characterized RaCBP in detail. The nucleotide sequence of the CBP gene of \textit{R. albus} NE1 is identical to that of the \textit{Rumal}_0187 gene. This mirrors a close phylogenetic relationship, as shown by a comparison of 16S rRNA sequences of both bacterial strains.\textsuperscript{34}
at higher concentrations. This behavior cannot be explained by the competitive inhibition model.\textsuperscript{40} Hence we predicted uncompetitive inhibition, in which D-glucose binds the ternary complex composed of the enzyme, D-glucose, and Glc1P to inhibit the enzyme reaction, in addition to competitive inhibition (Fig. 2).

The apparent kinetic parameters fitted well to the theoretical lines calculated by eq. (3) and eq. (4) (Fig. 2c). The calculated values were verified by fitting the experimental values to them (Fig. 5a). These results indicate that the competitive-uncompetitive model (Fig. 2) is applicable to inhibition by D-glucose for the reverse synthetic reaction of RaCBP. The kinetic parameters for the competitive model and the competitive-uncompetitive model are in common, other than $K_{i2}$, the dissociation constant for the quadruplet complex (the EPPQ complex in Fig. 2).

The $K_{iQ}$ value for the synthetic reaction of RaCBP was considerably lower than those of known enzymes ($K_{iA}$ for \textit{C. gilvus} CBP\textsuperscript{45}). This indicates that in the synthetic reaction of RaCBP, the formation of the enzyme-Glc1P complex (E + Q $\rightarrow$ EQ, Fig. 2) proceeds much more rapidly than the production of the reaction products from the enzyme-Glc1P-D-glucose complex (EQ $\rightarrow$ E + A + B, Fig. 2), because the very low $K_{iQ}$ indicates that $k_3$ was significantly higher than $k_3$. But the relationship between the low $K_{iQ}$ value and inhibition mechanism due to glucose is unclear.

The synthetic reaction of RaCBP is thought to be inhibited by D-glucose mainly in a competitive manner rather than an uncompetitive manner involving the formation of an inactive quadruplet complex, because the $K_{i3}$ value was significantly higher than $K_{i1}$ and $K_{i2}$ values. In uncompetitive inhibition, D-glucose, which secondly binds to the enzyme, might cause incorrect binding of the D-glucose bound to the acceptor site (the acceptor substrate), interrupting the reaction. It is possible that uncompetitive inhibition by D-glucose, proposed here, occurs even in CBPs from \textit{C. gilvus} and \textit{C. thermocellum}. The $K_{iQ}$ values, the dissociation constants for the enzyme-Glc1P complex, of CBPs from \textit{C. gilvus} and \textit{C. thermocellum} were 1.6- and 12.2-fold higher respectively than that of RaCBP.\textsuperscript{20,45} Hence D-glucose more efficiently binds to their Glc1P sites in a competitive manner than that of RaCBP, and presumably uncompetitive inhibition did not occur at the concentrations of D-glucose that were analyzed. \textit{T. maritima} CBP showed similar $K_{iQ}$ to RaCBP, and was mildly inhibited by D-glucose, like RaCBP.\textsuperscript{17} Its reverse synthetic reaction might also obey the competitive-uncompetitive inhibition model, although its kinetic mechanism was not fully investigated.

No complex structure of CBP with substrate at subsite $-1$, the Glc1P site, is available. But the amino acid residues surrounding subsite $-1$ of \textit{C. gilvus} CBP were predicted by superimposition of $N$-acetyl-D-glucosamine bound to subsite $-1$ of \textit{V. proteolyticus} chitobiose phosphorylase and the complex structures of \textit{C. gilvus} CBP with inhibitors (isofagomine and 1-deoxynojirimycin).\textsuperscript{31,32,46} These are essentially conserved between the two enzymes, although the orientation of the Arg residue (Arg362 of RaCBP and Arg343 of \textit{V. proteolyticus} chitobiose phosphorylase), which contributes to the interaction with the chemical group at the C2 position, is different due to a difference in the structures of the substrates. Isofagomine, lacking the OH group at the C2 position, is different due to a difference in the structures of the substrates.

Amino acid residues forming subsite $+1$, the D-glucose site, of
C. gilvus CBP (Gln165, Asp490, Glu649, Tyr653, Lys658, and Glu659) were clearly confirmed by structural analysis of the complex with β-glucose. These amino acid residues are also completely conserved in RaCBP. Hence, no amino acid substitution was found in the substrate binding site in RaCBP, although RaCBP showed different behavior in inhibition by β-glucose for the reverse synthetic reaction. Indirect interactions by amino acid residues around the substrate binding site might be involved in this phenomenon. Structural analysis of RaCBP is required for detailed understanding.

RaCBP showed high phosphorolytic activity only to cellobiose among the substrates tested, although it acted weakly on lactose. This indicates that RaCBP is highly specific to the β,1-4-glucosidic linkage, like other known enzymes. In the synthetic reaction, α-galactose, a 4-OH epimer of β-glucose, was not active at all (Table 1), consistently with the high specificity for 6-deoxy-β-glucose. These apparent kinetic parameters, \( k_{\text{cat}} \) for \( \text{Glc1} \text{C}_6 \text{C}_1 \) and \( k_{\text{m}} \) for \( \text{Glc1} \text{C}_6 \text{C}_1 \), were respectively observed for D-xylose. This indicates that the methyl group of 6-deoxy-β-glucose is essential for binding to the enzyme, as observed for the other enzymes.\(^{17,18,20,45}\) When the 2-OH position of β-glucose was substituted, the \( k_{\text{m}} \) and \( k_{\text{cat}} \) values respectively were observed for 3-deoxy-β-glucose, suggesting that the 6-ΟH group contributed only weakly to acceptor binding. In contrast, much higher and lower \( k_{\text{m}} \) and \( k_{\text{cat}} \) values respectively were observed for 3-xylene. This indicates that the methyl group of 6-deoxy-β-glucose is essential for binding to the enzyme, as observed for the other enzymes.\(^{17,18,20,45}\) The 2-ΟH position of β-glucose was substituted, the \( k_{\text{m}} \) and \( k_{\text{cat}} \) values significantly increased and decreased respectively. The configuration of 2-ΟH group is thought to be crucial for acceptor binding in RaCBP, as confirmed for the reported CBPs.\(^{17,18,20,45}\)

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References