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The transglycosylation reaction of cyclodextrin glucanotransferase is operated by a Ping-Pong mechanism

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Abstract

A new photometric assay of the disproportionation activity of cyclodextrin glucanotransferase (CGTase) using 3-ketobutylidene-β-2-chloro-4-nitrophenyl-maltopentaoside as the donor, proved that the transglycosylation reaction of CGTase was operated by a Ping-Pong Bi Bi mechanism. The values of the $k_{cat}/K_m^{acceptor}$ proved that the same configurations of free hydroxyl groups with those of D-glucopyranose at C2, C3 and C4 positions were required for the acceptors used by CGTase. The structure around C6 on acceptors was not essential for acceptor function, but it was recognized by CGTase, since the values of k_{cat}/K_m for D-xylose were smaller than that for D-glucose. The value of k_{cat}/K_m for maltose was about 20-times larger than that for D-glucose, indicating that at least two glucopyranosyl rings are recognized by the acceptor binding sites.

Key words: Amylolytic enzyme; Cyclodextrin glucanotransferase; Ping-Pong mechanism; Transglycosylation; 3KB-G5CNP

1. Introduction

Cyclodextrins, which have closed ring structures consisting of more than six D-glucose units linked by α-1,4-glucosidic bond, have potential uses in a variety of applications. Therefore, the cyclodextrin producing enzyme, cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19), is important in the food and pharmaceutical industries. CGTase produces cyclodextrins from starch and related α-1,4-glucans through an intramolecular transglycosylation reaction. Besides this cyclization reaction, the enzyme also catalyzes a coupling reaction (opening of the cyclodextrin rings and transfer of the resulting linear maltooligosaccharides to acceptors) and a disproportionation reaction (transfer of linear maltooligosaccharides to acceptors) through intermolecular transglycosylation reactions. Furthermore, CGTase has weak hydrolyzing activity.

Because of the similarity of the primary and tertiary structures between CGTases and α-amylases, the reaction mechanisms of both enzymes are thought to be similar [1–7]. The four conserved regions between CGTases and α-amylases (designated as the A-, B-, B′-, and C-region) have also been found in other amylolytic enzymes such as isoamylase and pullulanase [8,9]. These enzymes possessing the conserved regions have been classified into one family, α-amylase family, with the following characteristics [10]: (i) they act on α-glucosidic bonds; (ii) they split α-glucosidic bonds to produce α-anomeric saccharides or to form α-glucosidic linkages; (iii) they have four conserved regions in their primary sequences and have Asp, Glu, and Asp in the B-, B′-, and C-regions, respectively, as the essential amino acids for catalysis [11–16]. This family includes α-1,4-hydrolase, α-1,6-hydrolase, α-1,4-transglycosylase, and α-1,6-transglycosylase. Among them, CGTase typically catalyzes α-1,4-transglycosylation, and is a key enzyme for analyzing the reaction mechanism of the α-amylase family.

To analyze the reaction mechanism of CGTase, we developed a new photometric assay for CGTase activity. This assay is based upon the disproportionation reaction between a maltooligosaccharide with its non-reducing end blocked and with aglycon at its reducing end, 3-ketobutylidene-β-2-chloro-4-nitrophenyl-maltopentaoside (3KB-G5CNP), and suitable acceptors. Because 3KB-G5CNP is modified at the C4 hydroxyl group of its non-reducing end [17], CGTase cannot use it as an acceptor. Using this assay, we found that the intermolecular transglycosylation reaction of CGTase is operated by a Ping-Pong mechanism, and that the same configurations of hydroxyl groups with those of D-glucose at C2, C3, and C4 positions are required for acceptors used by CGTase. Furthermore, the acceptor binding site of CGTase can recognize at least two glucopyranosyl rings.

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Abbreviations: CGTase, cyclodextrin glucanotransferase; CNP, 2-chloro-4-nitrophenol; 3KB-G5CNP, 3-ketobutylidene-β-2-chloro-4-nitrophenyl-maltopentaoside.

2. Materials and methods

2.1. Chemicals and enzymes

3KB-G5CNP was purchased from Toyobo Co., Ltd. (Osaka, Japan). Amylose (the average degree of polymerization was 17) was a gift from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Soluble starch was purchased from E. Merck (Darmstadt, Germany). α-Glucosidase from *Saccharomyces* sp. was from Wako Pure Chemical Industry Ltd. (Osaka, Japan). β-Glucosidase from almonds was from Oriental Yeast (Tokyo, Japan). All other chemicals used were of reagent grade.

Recombinant CGTase, originated from alkalophilic *Bacillus* sp. #1011 [1] was produced by a protease-deficient mutant *Escherichia coli* ME8417 [*lon*::Tn*10*(*tet*^{1}) *thr leu lacY*], carrying the plasmid pTUE254 [15]. CGTase in the periplasm of the *E. coli* strain was extracted and purified as described previously [15]. The purity of the enzymes exceeded 90%, based upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2.2. Enzyme assay

All reactions were performed at 37°C in 10 mM sodium phosphate, pH 6.5. Starch-degrading activity was measured by the blue value method of Fuwa [18] with slight modifications using 0.3% soluble starch as the substrate. One unit of starch-degrading activity was defined as the amount of enzyme that gave a 1% decrease in absorbance at 660 nm per min. The acceleration effect of various acceptors on starch-degrading activity was analyzed by the method of Kitahata et al. [19] with slight modifications. Since the major degradation product from soluble starch and amylose catalyzed by *Bacillus* sp. #1011 CGTase is β-cyclodextrin [20], its formation was measured. The β-cyclodextrin-forming activity was assayed as described previously [20], using amylose (the average degree of polymerization was 17) as the substrate. The amounts of reducing sugar liberated by the hydrolyzing activity of the enzyme were measured by the methods of Somogyi and Nelson [21,22]. The coupling activity between γ-cyclodextrin and methyl-α-glucoside was assayed based upon the method of Thoma et al. [23] with modifications as described previously [20]. Disproportionation activity between 3KB-G5CNP and acceptors was assayed as follows: (i) reaction mixtures containing 3KB-G5CNP and an acceptor were incubated at 37°C with CGTase. (ii) At appropriate time intervals, 950 μl of the reaction mixture was mixed with 150 μl of 0.2 N hydrochloric acid to stop the reaction, and 200 μl of 0.5 M disodium hydrogenphosphate was then added. (iii) One milliliter of 10 mM sodium phosphate, pH 6.5, containing 1.5 units of α-glucosidase and 1.0 unit of β-glucosidase was added and the reaction mixtures were incubated for 1 h at 37°C. Subsequently, 100 μl of 1 M sodium carbonate was added to raise the pH of the reaction mixtures above 8.5. (iv) The degradation of 3KB-G5CNP was calibrated from the increases in absorbance at 400 nm, caused by CNP produced by α- and β-glucosidase treatment of the reaction mixture. The disproportionation activity of CGTase was calculated from the following equation:

$$\text{Disproportionation activity } (s^{-1}) = \{(A/s) \times V \times 1{,}000\}/\{16.8 \times [E] \times v\}$$

where A/s = absorbance increase per second at 400 nm; V = assay volume (ml), which was 2.4 in above procedure; 1,000 = conversion of liters to milliliters; 16.8 = millimolar extinction coefficient of CNP at 400 nm and pH > 8.5; [E] = enzyme concentration (M); v = pick up volume (ml), which was 0.95 in the above procedure.

Kinetic parameters of k_{cat} and K_m were determined by the nonlinear least-squares method with the Taylor expansion [24].

3. Results and discussion

To test whether CGTase can use 3KB-G5CNP as a substrate, they were mixed in the presence or absence of D-glucose as an acceptor, and incubated at 37°C (Fig. 1). In the absence of D-glucose, CGTase very slightly de-

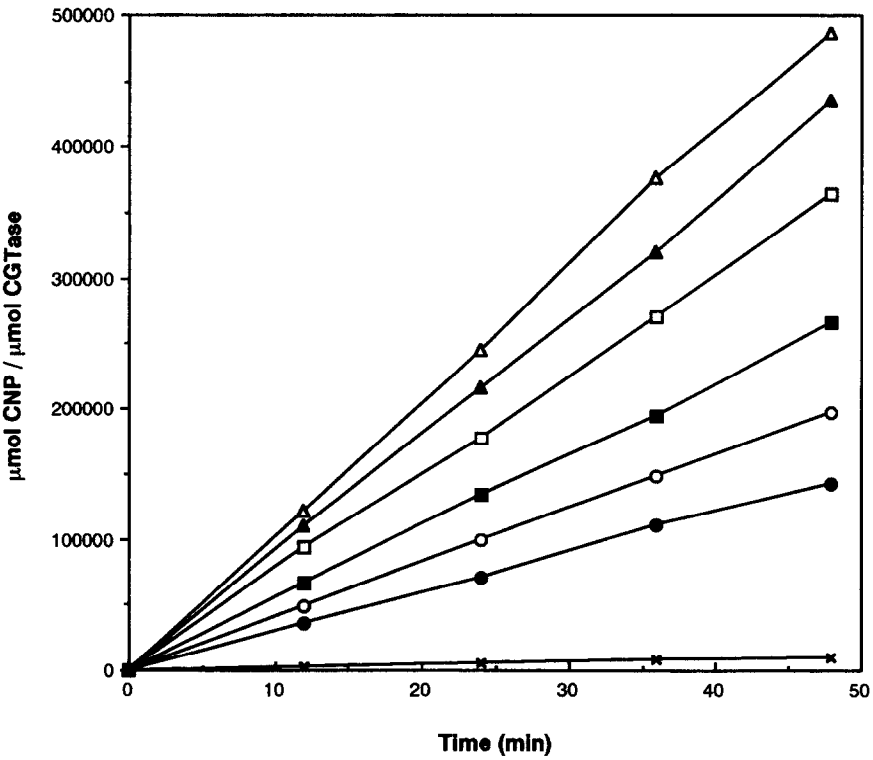

Fig. 1. The dependence of D-glucose concentrations on 3KB-G5CNP degradation. The assays were performed at 37°C in 10 mM sodium phosphate, pH 6.5, containing 1.5 mM 3KB-G5CNP and various concentrations of D-glucose. The concentrations of CGTase were 0.1 μg/ml in the absence of D-glucose and 0.004 μg/ml in the presence of D-glucose. The final concentrations of D-glucose were: X, none; ●, 3.0 mM; ○, 4.5 mM; ■, 7.5 mM; □, 13.5 mM; ▲, 21 mM; △, 45 mM.

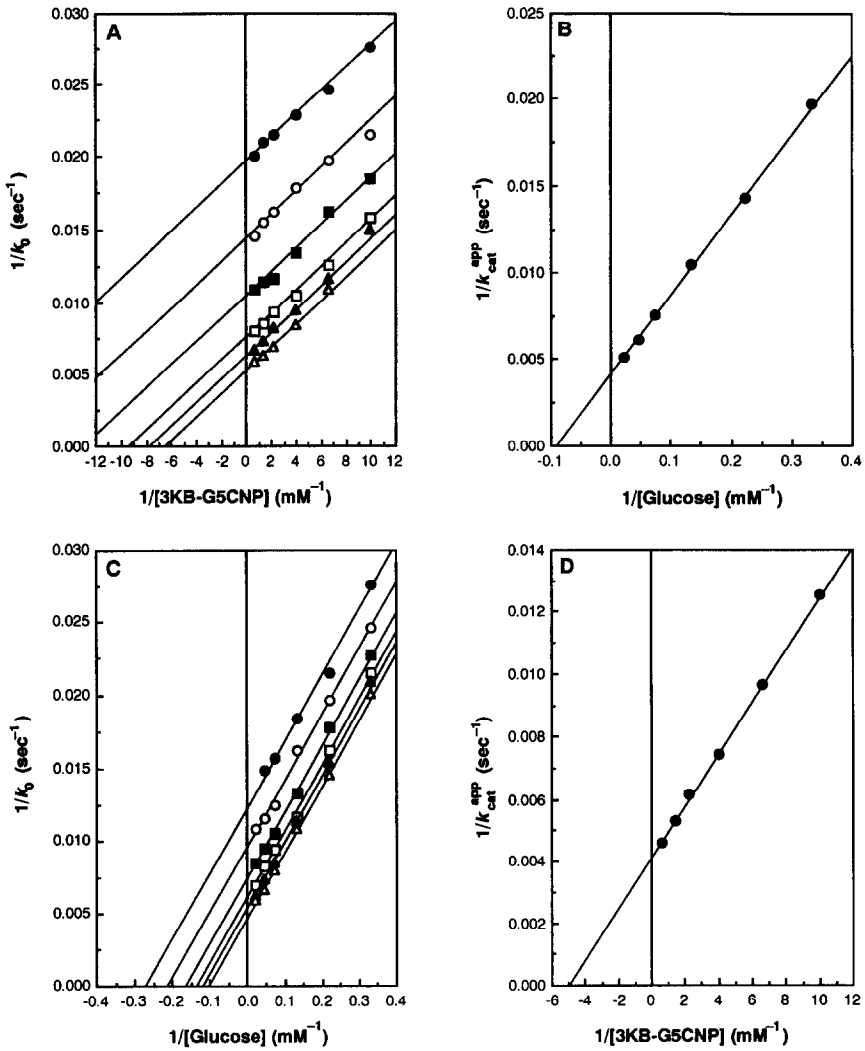

Fig. 2. The disproportionation reaction of CGTase is operated by Ping-Pong Bi Bi mechanism. (A) Double reciprocal plots at varied concentrations of 3KB-G5CNP with fixed concentrations of D-glucose. The concentrations of D-glucose were ●, 3.0 mM; ○, 4.5 mM; ■, 7.5 mM; □, 13.5 mM; ▲, 21 mM; △, 45 mM. (B) Secondary plot of $1/k_{cat}^{app}$ from (A) versus the reciprocal of the D-glucose concentration. (C) Double reciprocal plots at varied concentrations of D-glucose with fixed concentrations of 3KB-G5CNP. The concentrations of 3KB-G5CNP were ●, 0.10 mM; ○, 0.15 mM; ■, 0.25 mM; □, 0.45 mM; ▲, 0.70 mM; △, 1.5 mM. $1/k_{cat}^{app}$ vs. 1/[3KB-G5CNP] plot. (D) Secondary plot of $1/k_{cat}^{app}$ from (C) vs. the reciprocal of the 3KB-G5CNP concentration.

graded 3KB-G5CNP. This suggests that CGTase does not hydrolyze 3KB-G5CNP very well and that it does not function well as an acceptor for the disproportionation reaction. In contrast, the production of CNP, produced from degraded 3KB-G5CNP by α- and β-glucosidase digestion, was increased in a linear fashion according to the reaction time in the presence of D-glucose. The rates for the production of CNP were increased in proportion to the D-glucose concentration, indicating that the degradation rate of 3KB-G5CNP by CGTase depends on the concentration of the acceptor. The initial velocities for the disproportionation reaction were determined, in which the 3KB-G5CNP and D-glucose concentrations were varied independently (Fig. 2). When various concentrations of 3KB-G5CNP were plotted with different concentrations of D-glucose as the fixed-concentration substrate, parallel double reciprocal plots were obtained (Fig. 2A). The replots of the intercepts ($1/k_{cat}^{app}$) of these lines against the reciprocal of D-glucose concentrations were linear (Fig. 2B). When various concentrations of D-glucose were plotted with different concentrations of 3KB-G5CNP as the fixed-concentration substrate, parallel double reciprocal plots were also obtained (Fig. 2C). The replots of the intercepts ($1/k_{cat}^{app}$) of these lines against the reciprocal of 3KB-G5CNP concentrations were linear (Fig. 2D). Similar results were obtained when maltose was used instead of D-glucose as the acceptor (data not shown). These results indicate that the disproportionation reaction of CGTase is operated by a Ping-Pong Bi Bi mechanism. The kinetic parameters

Table 1
Kinetic parameters of *Bacillus* sp. #1011 CGTase[a]

Starch-degrading activity (units/mg)	2,170
β-Cyclodextrin-forming activity[b]	
k_{cat} (s^{-1})	30.5 ± 0.6
K_m (μM)	57.4 ± 3.8
α-Cyclodextrin hydrolyzing activity	
k_{cat} (s^{-1})	2.65 ± 0.008
K_m (mM)	0.57 ± 0.05
Coupling activity	
k_{cat} (s^{-1})	67.8 ± 1.6
$K_m^{\gamma\text{-cyclodextrin}}$ (mM)	0.119 ± 0.008
$K_m^{\text{methyl-α-D-glucoside}}$ (mM)	16.9 ± 1.4
Disproportionation activity	
D-Glucose	
k_{cat} (s^{-1})	244 ± 6
$K_m^{\text{3KB-G5CNP}}$ (mM)	0.204 ± 0.015
$K_m^{\text{D-glucose}}$ (mM)	11.3 ± 0.4
Maltose	
k_{cat} (s^{-1})	203 ± 3
$K_m^{\text{3KB-G5CNP}}$ (mM)	0.164 ± 0.005
K_m^{maltose} (mM)	0.487 ± 0.019

[a] All assays were performed at 37°C in 10 mM sodium phosphate, pH 6.5.
[b] Amylose (the average degree of polymerization of amylose was 17) was used as the substrate.

for starch-degrading, α-cyclodextrin-hydrolyzing, β-cyclodextrin-forming, coupling, and disproportionation activities were calculated and are summarized in Table 1. The k_{cat} values for disproportionation activity were larger than those for hydrolyzing, β-cyclodextrin-forming, and coupling activity. This suggests that the main reaction catalyzed by CGTase is disproportionation, not cyclization. Cyclodextrin will be produced only when the non-reducing end of cleaved amylose bound to the enzyme is recognized by the acceptor binding site.

Acceptor specificity of CGTase has been proposed by the acceleration effects of acceptors on starch-degrading activity [19], although the specificity could not be evaluated quantitatively from this effect. The acceleration effects of acceptors on starch-degrading activity are summarized in Table 2. The activity was accelerated by L-sorbose, D-glucose, methyl-α-glucoside, and sucrose. D-Xylose and maltose had little effect on acceleration, whereas D-galactose, 3-*O*-methyl-D-glucose, D-mannose and D-fructose did not have significant acceleration effects.

As shown in Fig. 2, the disproportionation reaction of CGTase is operated by a Ping-Pong Bi Bi mechanism. The values of $k_{cat}/K_m^{\text{acceptor}}$ for the disproportionation activity of CGTase were independent of the 3KB-G5CNP concentration. Since k_{cat}/K_m values are related to the total binding energy of an enzyme and a substrate [25], the substrate specificity of enzymes can be evaluated quantitatively from the value of k_{cat}/K_m. We evaluated the acceptor specificity of this CGTase from the values of $k_{cat}/K_m^{\text{acceptor}}$ (Table 2). The sugars that accelerated the starch-degrading activity of CGTase, had relatively high k_{cat}/K_m values. In contrast, the disproportionation activities for sugars that did not accelerate starch-degrading activity were too low and so their k_{cat}/K_m values could not be determined. The values of k_{cat}/K_m for D-glucose, methyl-α-glucoside, and L-sorbose indicated that they were good acceptors for CGTase. The configurations of hydroxyl groups at C3, C4, and C5 positions of L-sorbose are the same as those at C4, C3, and C2 positions of D-glucose, respectively. D-Mannose and D-galactose, which have different configurations at the C2 and C4 hydroxyl groups from those of D-glucose, respectively, hardly used as acceptors. D-Fructose and 3-*O*-methyl-D-glucose also could not be used as acceptors. These results show that the same configurations of free hydroxyl groups at C2, C3 and C4 positions as those of D-glucopyranose seem to be required for acceptors used by CGTase. This conclusion is consistent with the assumption derived from the acceleration effect of acceptors on the starch-degrading activity [19].

D-Xylose, which has the same configurations of hydroxyl groups at positions C2, C3 and C4 as those of D-glucose except for C6, can be used as an acceptor, suggesting that the structure around C6 of D-glucose is not essential for binding to the acceptor binding site of CGTase. The value of k_{cat}/K_m for D-xylose, however, was 14 times smaller than that for D-glucose, suggesting that the acceptor binding site of CGTase recognizes the structure around C6 of D-glucose as well as the hydroxyl groups at positions C2, C3, and C4. The k_{cat}/K_m value for

Table 2
Acceleration effect of various acceptors upon starch-degradation and the k_{cat}/K_m values of disproportionation[a]

Acceptor	Acceleration effect[b]	Disproportionation activity[c] k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Methyl-α-D-glucoside	3.2	166
L-Sorbose	6.4	35.4
D-Glucose	4.8	21.0
D-Xylose	1.8	1.47
D-Galactose	1.1	–[d]
3-*O*-Methyl-D-glucose	1.0	–[d]
D-Fructose	1.0	–[d]
D-Mannose	1.0	–[d]
Maltose	1.7	420
Sucrose	2.9	5.53

[a] All assays were performed at 37°C in 10 mM sodium phosphate, pH 6.5.
[b] Rates of starch-degrading activity in the presence of acceptors at a final concentration of 0.2 mM relative to the activity in the absence of acceptors.
[c] The assay was performed using a fixed concentration of 3KB-G5CNP (0.5 mM) and varied concentrations of acceptors.
[d] Could not be determined correctly because the activity was too low.

maltose was 20 times larger than that for D-glucose, indicating that the acceptor binding site of CGTase can recognize at least two glucopyranose. The value of k_{cat}/K_m for sucrose was about four times smaller than that for D-glucose suggesting that the fructofuranocyl ring of sucrose inhibits acceptor binding to the acceptor binding sites of CGTase.

Because of their primary and tertiary structural similarities, CGTases and α-amylases are considered to be of the α-amylase family, although the former is transferase and the latter is hydrolase [10]. This family also includes α-1,6-hydrolases and α-1,6-transglycosylases such as pullulanase [9] and branching enzyme [26]. It has been proposed that neopullulanase catalyzes α-1,4-hydrolysis, α-1,6-hydrolysis, α-1,4-transglycosylation, and α-1,6-transglycosylation reactions at the same active site through the same reaction mechanism [10]. Some α-amylases have significantly high level of transglycosylation activity in the presence of high substrate concentrations. Furthermore, the W84L mutation of α-amylase from *Saccharomycopsis fibuligera* induces considerably high transglycosylation activity [27]. It has been proposed that α-amylase splits α-1,4-glucosidic bonds through an oxocarbonium ion-like intermediate [3]. Hydrolysis will occur when this intermediate is attacked nucleophilically by a water molecule. Although the precise reaction mechanism for the transglycosylation reaction of these hydrolases remains unclear, it would also be operated by a Ping-Pong mechanism, because the reaction mechanisms of α-amylase family are thought to be similar. Transglycosylation will occur when the oxocarbonium ion intermediate is attacked by an intact substrate instead of a water molecule.

The high transglycosylation and relatively low hydrolyzing activities of CGTase may be due to a difference in the physicochemical environment at the active center. The three-dimensional structures of α-amylases and CGTases indicate that many residues, which are proposed to be involved in substrate binding and catalysis, are conserved between them [3–7]. Four aromatic residues (Phe^{183}, Tyr^{195}, Phe^{259}, and Phe^{283} of *Bacillus* sp. #1011 CGTase), however, are present at the active center in CGTases, but not in α-amylases. These residues may be involved in the characteristics that differ between CGTases and α-amylases [5].

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